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Type 1 Diabetic Heart: Examination of Mitochondrial Structure and MicroRNAs

Dharendra Thapa

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Type 1 Diabetic Heart: Examination of Mitochondrial Structure and MicroRNAs

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Dissertation submitted to the School of Medicine at
West Virginia University in partial fulfillment of the
requirements for the degree of

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in
Exercise Physiology

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ABSTRACT

Type 1 Diabetic Heart: Examination of Mitochondrial structure and microRNAS

Dharendra Thapa

Cardiac complications such as diabetic cardiomyopathy are the leading cause of morbidity and mortality in patients with diabetes mellitus. Dysfunctional mitochondria, an effect associated with cardiomyopathy, are central in the pathogenesis of type 1 diabetes mellitus. Cardiac mitochondria are comprised of two spatially located mitochondria including mitochondria located beneath the sarcolemma, termed subsarcolemmal mitochondria (SSM) and those located in between myofibrils, termed interfibrillar mitochondria (IFM). Mitochondrial subpopulations have been shown to respond differently to pathological and physiological stimuli as reported in a review published by our laboratory. IFM mitochondria are most impacted in a type 1 diabetic setting. Proteomic alterations in cardiac mitochondria during a diabetic insult reveal impact primarily on IFM on nuclear-encoded mitochondrial proteins, a finding that has been previously reported by our laboratory. Alterations of proteins encoded by the mitochondrial genome have not been observed in our proteomics. Further, regulation of nuclear-encoded proteins by microRNAs (miRNAs) has been previously reported by our laboratory. MiRNAs are 22 nucleotide long post-transcriptional regulators with a 7 nucleotide seeding region specific to complementary sequences in the mRNA. More than 30% of all proteins are regulated by miRNAs and one miRNA has the potential to regulate the expression of multiple proteins. The potential regulation of mitochondrial genome encoded proteins by miRNAs has yet to be investigated in mitochondrial subpopulations during diabetes. Among the altered nuclear encoded proteins in type 1 diabetic IFM is structural protein known as mitofilin. Mitofilin is an inner mitochondrial membrane structural protein, well established for its role in maintaining cristae morphology and structure. It is a central component of the mitochondrial contact site and cristae organizing system (MICOS) complex. Interactions of mitofilin with outer and inner membrane proteins have been reported to be crucial for mitochondrial membrane organization, cristae integrity and inner membrane architecture. Moreover, MICOS has been shown to function in concert with ATP synthase dimers. However, association of mitofilin with ATP synthase subunits is not known. Moreover, literature examining association of mitofilin and regulation of mitochondrial genome by miRNAs in type 1 diabetic insult is sparse. Also, the impact of diabetes mellitus on mitofilin protein interactions, mitochondrial structure and function are currently unclear. It is specifically unknown whether overexpression of mitofilin aids in alleviating complications associated with diabetic cardiomyopathy. The goal of the present studies was to determine novel association of mitofilin and the impact of mitofilin overexpression upon mitochondrial structure and function. Further, regulation of mitochondrial

genome by mitochondrial miRNAs (mitomiRs) has been investigated. The overall hypothesis of this application is that alterations of cristae morphology, inner membrane organization and mitochondrial dysfunction observed during type 1 diabetic insult are associated with decrements in mitofilin content as well as translational regulation of mitochondrial encoded proteins due to altered levels of mitochondrial miRNAs (mitomiRs). Type 1 diabetes mellitus was induced in five weeks old mice with multiple low dose injections of streptozotocin (STZ) for five consecutive days. Five weeks post hyperglycemic onset, hearts were excised and mitochondrial subpopulations isolated for further studies. Using a gel based technique, mitochondrial proteins immunoprecipitated with mitofilin were subjected to LC-ESI-MS analysis. Proteins from all electron transport chain complexes, structural proteins and proteins involved in protein import were identified in an immunoprecipitated complex. Association of mitofilin with F_0 -ATP synthase subunit b (ATP5F1) was decreased in the diabetic IFM when compared with control. Moreover, interaction of mitofilin with coiled-coil-helix coiled-coil-helix domain 3 (CHCHD3) trended towards decreased in diabetic IFM. A transgenic mouse line overexpressing mitofilin was generated and utilized to investigate the role of mitofilin overexpression in mitochondrial structure and function. Restoration of ejection fraction and fractional shortening was observed in mitofilin diabetic mice as compared to wild-type controls ($P < 0.05$ for both). Decrements observed in electron transport chain (ETC) complexes I, III, IV and V activities, state 3 respiration, lipid peroxidation as well as mitochondria membrane potential in type 1 diabetic IFM were restored in mitofilin diabetic mice ($P < 0.05$ for all). Qualitative analyses of electron micrographs revealed restoration of mitochondrial cristae structure in mitofilin diabetic mice as compared to wild-type controls. Furthermore measurement of mitochondrial internal complexity using flow cytometry displayed significant reduction in internal complexity in diabetic IFM which was restored in mitofilin diabetic IFM ($P < 0.05$). No significant changes in mitochondrial dynamic regulating proteins or mitochondrial DNA content were observed. Examination of mitochondrial miRNAs was performed using microarray technology coupled with cross-linking immunoprecipitation and next generation sequencing, we identified a functional pool of mitochondrial microRNAs, termed mitomiRs that are redistributed in spatially-distinct mitochondrial subpopulations in an inverse manner following diabetic insult. Redistributed mitomiRs displayed distinct interactions with the mitochondrial genome requiring specific stoichiometric associations with RISC constituents argonaute-2 (Ago2) and fragile X mental retardation-related protein 1 (FMR1) for translational regulation. In the presence of Ago2 and FMR1, redistribution of mitomiR-378 to the IFM following diabetic insult led to down regulation of mitochondrially-encoded F_0 component (ATP6). Next generation sequencing analyses identified specific transcriptome and mitomiR sequences associated with ATP6 regulation. Overexpression of mitomiR-378 in HL-1 cells resulted in its accumulation in the mitochondrion and down-regulation of functional ATP6 protein, while antagomir blockade restored functional ATP6 protein and contractile function. The results reveal the requirement of RISC constituents in the mitochondrion for functional mitomiR translational regulation and provide a connecting link between diabetic insult and ATP synthase function. Taken together, the studies highlighted

above show that mitochondrial dysfunction observed during type 1 diabetic insult could be due to multiple reasons including decrements in mitofilin content and dysregulation of mitomiRs in IFM subpopulations. Overexpression of mitofilin targeted to restore mitochondrial structure and utilization of antagomir target at mitomiR-378 both restored mitochondrial functionality and cardiac contractile function during type 1 diabetic insult.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AGO2	Argonaute 2
AmBIC	Ammonium Bicarbonate
ARE	AU RICH Elements
ATP	Adenosine Triphosphate
ATP5F1	ATP Synthase Subunit b
BN-PAGE	Blue Native PAGE
CAD	Coronary Artery Disease
CHCHD3	Coiled-Coil-Helix-Coiled-Coil-Helix Domain 3
CHCM1	Coiled-Coil-Helix Cristae Morphology 1
CLIP	Crosslinked Immunoprecipitation
CMV	Cytomegalovirus
COX	Cytochrome c Oxidase
DM	Diabetes Mellitus
DNA	Deoxyribo Nucleic Acid
Drp1	Dynamin-Related Protein 1
DTT	Dithiothreitol
EDP	End Diastolic Pressure
EF	Ejection Fraction
ET-1	Endothelin -1
ETC	Electron Transport Chain

FCJ1	Formation of Crista Junction 1
FPKM	Fragments per Kilobase of Exon per Million
FSC	Forward Scatter
FXR1	Fragile X Mental Retardation-Related Protein 1
HNSCCs	Head and Neck Squamous Cell Carcinomas
IA	Iodoacetamide
IFM	Interfibrillar Mitochondria
IMM	Inner Mitochondrial Membrane
IPA	Ingenuity Pathway Analysis
LV	Left Ventricle
Mfn	Mitofusin
MIB	Mitochondrial Intermembrane Space Bridging
MINOS	Mitochondrial Inner Membrane Organizing System
miRNAs	MicroRNAs
mitomiRs	Mitochondrial MicroRNAs
MitOS	Mitochondrial Organizing Structure
MnSOD	Manganese Superoxide Dismutase
MPTP	Mitochondrial Permeability Transition Pore
OPA1	Optic Atrophy 1
OXPHOS	Oxidative Phosphorylation
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
RISC	RNA Induced Silencing Complex

ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
SOD	Superoxide Dismutase
SSC	Side Scatter
SSM	Subsarcolemmal Mitochondria
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Ψ_m	Membrane Potential

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SPECIFIC AIMS

Diabetic cardiomyopathy, characterized by contractile dysfunction, independent of atherosclerosis, is the leading cause of heart failure in patients with diabetes mellitus (5). Dysfunctional mitochondria are central to the pathogenesis of diabetes mellitus (4, 11, 12). The inner mitochondrial membrane (IMM) is comprised of the inner boundary membrane and cristae membranes (9, 15). Mitofilin, a relatively unstudied inner mitochondrial membrane structural protein controls cristae morphology and is a central component of the mitochondrial inner membrane organizing system or mitochondrial organizing structure (MitOS) (7, 8, 13) more recently named as mitochondrial contact site and cristae organizing system (MICOS) (10). Moreover, interactions of mitofilin with outer and inner membrane proteins have been reported to be crucial for membrane organization and protein biogenesis (1, 6, 13, 14). MICOS has also been shown to function in concert with ATP synthase dimers (7) and results from our laboratory reveal novel interactions of mitofilin with ATP synthase F₀ subunits. Study of mitochondria in cardiac tissue is complicated by the presence of two spatially distinct subpopulations, subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). Proteomic studies show a significant decrease of mitofilin in diabetic IFM (2) and other key proteins in the electron transport chain complexes (ETC). MicroRNAs (miRNAs) have been shown to regulate nuclear encoded proteins (3) but translational regulation of mitochondrial-genome encoded proteins have not been examined yet. There is **a gap in knowledge** as to examining the mitochondrial mechanisms affected by loss of mitofilin and regulation of mitochondrially-encoded proteins by miRNAs. Moreover, literature examining these mechanisms in type 1 diabetic insult is sparse. Also, the impact of diabetes mellitus on mitofilin protein interactions, mitochondrial structure

and function are currently unclear. It is specifically unknown whether overexpression of mitofilin aids in alleviating complications associated with diabetic cardiomyopathy.

Our **long term goal** is to better understand the mechanisms involved in the pathogenesis of diabetes mellitus for developing therapeutic interventions to protect against diabetic cardiomyopathy. **The objectives of this application are:** (1) to determine the impact of type 1 diabetes mellitus on mitofilin interactions with MINOS components and investigate novel associations of mitofilin with ATP synthase F0 subunits in mitochondrial subpopulations, (2) to determine the novel therapeutic benefit of mitofilin overexpression in mitochondrial morphology and cardiac function during a diabetic insult, and (3) examine and investigate mitochondrial miRNAs and their potential roles in translational regulation of the mitochondrial genome-encoded proteins during type 1 diabetes mellitus.

The central hypothesis of this application is that alterations of cristae morphology, inner membrane organization and mitochondrial dysfunction observed during type 1 diabetic insult are associated with decrements in mitofilin content as well as translational regulation of mitochondrial encoded proteins due to altered levels of mitochondrial miRNAs (mitomiRs). These effects are more pronounced in the diabetic IFM and influences cardiac contractile processes. Our **rationale** for the proposed research is based on the absolute importance of preserving IMM morphology and mitochondrial genome encoded proteins to attenuate mitochondrial dysfunction observed during type 1 diabetic insult. We anticipate being able to identify morphological and functional alterations in mitochondrial subpopulations and investigate areas at risk. The outcomes of this research application will provide insight in understanding the importance of preserved mitochondrial structure as well as potential roles of mitomiRs in regulating mitochondrial genome during diabetic cardiomyopathy, thus, further

aiding in the development of therapeutic interventions geared towards mitochondrial subpopulations at risk during type 1 diabetes mellitus. The objectives and central hypothesis of this application will be tested by pursuing the following specific aims:

Specific Aim I: Determine the impact of type 1 diabetes mellitus on interaction of mitofilin with the MINOS components and investigate novel associations of mitofilin with ATP synthase F0 subunits.

In order to address Specific aim I, we will utilize immunoprecipitation in conjunction with mass spectrometry to identify novel proteins pulled down with mitofilin. Our **working hypothesis** is that type 1 diabetes mellitus will decrease mitofilin content resulting in damaged mitochondrial structure and decreased ATP synthase activity as a result of decreased association of mitofilin with MINOS components and ATP synthase F0 subunits respectively. The effects will be more pronounced in IFM.

Specific Aim II: Evaluate the therapeutic benefit of mitofilin overexpression in mitochondrial morphology, function and cardiac function, following a type 1 diabetic insult. Further, determine if the effect is subpopulation specific.

So as to address this aim, we will utilize a novel mitofilin over expression transgenic animal generated in our laboratory. Further, we will examine mitochondrial morphology, dynamics, DNA content, functionality and ultimately cardiac contractile function during type 1 diabetic insult. Our **working hypothesis** is that overexpression of mitofilin will preserve mitochondrial and cardiac function through improved cristae morphology, and mitochondrial function during type 1 diabetes mellitus, particularly in the IFM.

Specific Aim III: Examine and investigate mitochondrial miRNAs and their potential roles in translational regulation of the mitochondrial genome-encoded proteins during type 1 diabetes mellitus.

Regulation of nuclear encoded genome proteins by miRNAs have been previously studied and reported by our laboratory. However, examination and presence of miRNAs in the mitochondrial subpopulations and their potential role in regulating mitochondrial genome-encoded proteins have not been studied. In order to assess this Specific Aim III, we will conduct microarrays experiments in control and diabetic cardiac subpopulations and analyze the miRNAs that are altered in type 1 diabetic phenotype. Our **working hypothesis** is that the miRNA profile will be altered in the diabetic IFM (subpopulation more affected by the disease phenotype) which will lead to decrements in mitochondrially-encoded proteins and hence function. Further, targeting of altered miRNAs by anatgomir treatment will lead to restoration of protein and associated mitochondrial functionality.

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Chapter 1:

Literature Review

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a pathological condition characterized by lack of insulin (Type 1 DM) or resistance to insulin (Type 2 DM) production. Type 1 DM accounts for 10% of all cases and type 2 DM is the more prevalent form accounting for 90% of all cases (141). DM is characterized by chronic hyperglycemia which is associated with damage and failure of different organs including the heart, eyes, kidney and blood vessels. If left untreated, both types of DM manifest various complications and ultimately result in death. Lesser known types of DM constitute gestational diabetes that occurs during pregnancies and DM resulting from genetic defects, surgeries, medicine as well as other pancreatic diseases. A fasting blood glucose level over 126 mg/dl suggests impaired glucose tolerance and is the criterion used to diagnose DM (155).

1.1a Type 1 DM

Type 1 DM (T1DM) also referred to as insulin dependent DM is caused by an absolute deficiency of insulin as a result of autoimmune destruction of pancreatic beta cells (81). Because of its majority of diagnoses occurring in children and young adults (19) it is also known as juvenile-onset diabetes. In addition to genetic predisposition, other environmental factors like viral infections, low birth weight, parental age and nutritional factors have also been associated with the development of T1DM (2). The main course of treatment used for T1DM is to monitor blood glucose level regularly and inject with artificial insulin prior to eating. Insulin deficiency as observed during T1DM increases ketone bodies in the liver and causes diabetic ketoacidosis, a metabolic complication characterized by hyperglycemia, ketosis and acidosis (48) which is fatal

if left untreated (32). Patients with T1DM are also prone to other autoimmune disorders like Graves' disease, Addison's disease, vitiligo, autoimmune hepatitis and pernicious anemia (1). All these manifestations observed during T1DM are associated with hyperglycemic environment observed during DM and there is no known prevention of T1DM.

1.1b Significance

Cardiac failure is the leading cause of mortality in patients with diabetes mellitus in the United States (63). In 2000, over 171 million people worldwide were suffering from diabetes mellitus, with an estimate of 366 million by the year 2030 (172). Over 40% of the population 20 years or older in United States are hyperglycemic (35). According to the National Diabetes Statistics Report, as of 2012, 29.1 million Americans are diabetic and the total direct and indirect cost of diagnosed DM is \$245 billion. These staggering statistics implicate the necessity for investigating potential therapeutic interventions and vindicates the importance of studying diabetic cardiomyopathy, an increasingly prevalent diagnosis in heart failure (13). Moreover, mitochondrial dysfunction is central in the pathogenesis of diabetic cardiomyopathy due to its role in ATP production for cardiac contractile processes and electrolytes transport.

1.2 Diabetic Cardiomyopathy

Diabetic cardiomyopathy, characterized by contractile dysfunction independent of atherosclerosis, is the leading cause of heart failure in patients with diabetes mellitus (27). A number of clinical, epidemiological, pathological and animal studies have examined and

suggested the existence of diabetic heart disease irrespective of hypertension and coronary artery disease (CAD) (68, 113, 132, 137). In a clinical setting, Rubler et. al (137) was the first to examine and acknowledge the presence of diabetic cardiomyopathy in diabetic patients with congestive heart failure without coronary atherosclerosis and hypertension. Existence of diabetic cardiomyopathy was further strengthened by a study examining four diabetic patients without CAD that showed an increase left ventricle (LV) end diastolic pressure (EDP), decreased LV compliance and decreased ejection fraction (EF) (132). Furthermore, studies utilizing type 1 diabetic rat models resistant to atherosclerosis showed decreased contractile performance implicating diabetic cardiomyopathy (84, 159). Hence, these studies present diabetic cardiomyopathy as a distinct clinical entity that is solely associated with ventricular dysfunction in the absence of hypertension or CAD. Further characterization of diabetic cardiomyopathy reveals several dysfunctions in the diabetic heart including contractile dysfunction, cardiac structural changes, and oxidative stress (51, 141).

1.2a Cardiac contractile dysfunction

Assessment of diastolic dysfunction relies on doppler studies of transmitral inflow that measures mitral inflow velocities, deceleration time, and isovolumic relaxation time, and assessing flow patterns (51). Diastolic dysfunction has been widely reported in both diabetic animals (62, 140) and patients (129, 130) without any other factors contributing to heart disease. A study examining influence of diabetes on contractile performance of rat myocardium show diastolic dysfunction characterized by prolongation of relaxation and considerable slowing in relaxation velocity (21). Similarly, perfused diabetic rat hearts showed prolonged isovolumic

relaxation time and increases in later mitral inflow velocity and LV EDP (85). Assessment of diastolic function in thirty diabetic male rats in comparison with control showed significant decrements in early to late diastolic mitral inflow velocity ratio and isovolumic relaxation time (44). The authors suggest that the presence of diastolic dysfunction in diabetic hearts might relate to uncoupling of the contractile apparatus without increments in chamber thickness. A study comprising eighty seven young T1DM patients without known cardiac disease displayed reduced early peak mitral velocity, increased late peak mitral velocity, and prolonged deceleration time and isovolumic relaxation time compared to controls (138).

DM has been associated with systolic dysfunction comprising reduction in heart rate, systolic blood pressure, and fractional shortening (70, 74, 85, 174). Also, reduction in systolic LV pressure has been observed in papillary muscle isolated from type 1 diabetic mouse hearts (156). However, association of LV systolic dysfunction with DM has not been uniformly reported (58, 110, 129). Although diastolic dysfunction is observed during DM, reports of systolic dysfunction are varied. This discrepancy could be due to less sensitive techniques utilized to assess systolic dysfunction. Advancements or more sensitive techniques for systolic assessment such as strain, strain rate, and myocardial tissue Doppler velocity can indeed detect systolic abnormalities in diabetic patients (52).

1.2b Structural changes in diabetes

Structural changes, focusing on fibrosis, have been reported in both animals (78, 154) and humans (96, 118, 152) in parallel with functional changes of diabetic heart disease, in the absence of hypertension and CAD. Extracellular fibrosis and abundant TGF- β 1 receptor II in LV

myocytes were observed in 15 wk old diabetic rats compared with control suggesting LV fibrosis in the early stage of the disease (113). In an autopsy study comprising 9 diabetic hearts, six with heart failure, Regan et al. reported increased interstitial fibrosis in diabetic hearts (132). Similar morphological alterations were observed by Nunoda et al. who reported a higher percentage of interstitial fibrosis in diabetic patients without hypertension or CAD (121). Mechanisms involved in fibrosis accumulation in the diabetic heart are not entirely elucidated. However, increased levels of plasma endothelin-1 (ET-1) have been associated with fibroblast accumulation in diabetic hearts (171). Further, enhanced levels of protein kinase C beta-1 activation have also been observed with increased fibrosis in streptozotocin (STZ) diabetic hearts (168). Hence, fibrosis plays a crucial role in the manifestation of diabetes induced cardiac dysfunction in both animals and humans.

1.2c Oxidative stress

Oxidative stress can be defined as an imbalance between reactive oxygen species (ROS) generation and antioxidant defense. Activation of renin-angiotensin system has been implicated in increased oxidative damage and development of diabetic cardiomyopathy during DM (45, 51, 59). Hyperglycemia is the primary cause of diabetic cardiomyopathy. Increased ROS production has been implicated to augment hyperglycemia induced activation of protein kinase C isoforms, formation of advanced glycation end products as well as increased flux of glucose through the aldose reductase pathways (22, 91) contributing to the development of cardiac complications in DM. Moreover, hyperglycemia has been suggested to decrease antioxidant levels in the diabetic hearts making it prone to ROS damage (135). Furthermore, cardiac overexpression of

mitochondrial superoxide dismutase (Sod2) in the type 1 diabetic heart has been shown to reverse altered mitochondrial structure and function (142). Mitochondria are the major source of ROS production in the cardiomyocytes. Previous work from our laboratory have shown increased level of ROS production and oxidative damage in STZ induced T1DM (38).

1.3 STZ mouse model of diabetic cardiomyopathy

Utilization of rodent models for investigating diabetic cardiomyopathy is beneficial because of their resistance to atherosclerosis. This allows investigator to examine diabetic cardiomyopathy dysfunction without the confounding effects of atherosclerosis. STZ, a glucosamine-nitrosourea was first utilized for destruction of cancerous pancreatic β -cells and then for induction of diabetes in rodent models (3). Because of its similarities to glucose, it is taken up by GLUT2 glucose transporter which is abundantly found in β -cells, causing DNA alkylation (139, 167). The DNA damage further leads to activation of poly ADP-ribosylation that causes diminished cellular NAD⁺ and ATP. Further, STZ induced enhanced ATP dephosphorylation offers substrate for xanthine oxidase to form superoxide radical. Finally, this increased expression of ROS causes necrosis of β -cells diminishing insulin production (139). Our laboratory utilizes the protocol established by the Animal Model of Diabetic Complications Consortium (AMDCC). AMDCC recommends multiple low-dose (50mg/kg) of STZ injection over five consecutive days. Generally, mice will develop fasting hyperglycemia 7-14 days after the first injection of STZ (23). Our laboratory and others have reported induction of diabetic cardiomyopathy, including systolic and diastolic dysfunction after STZ treatment (8, 38, 119, 151). STZ model is widely used because of the ability to induce diabetes in any rodent strains.

Also, diabetes can be induced at any age allowing the investigation of diabetic heart at various stages in the life cycle of the organism (23).

1.4 Mitochondrial subpopulations

With the advent of advanced imaging techniques, insight into cellular and organellar structure of the cardiomyocyte has provided a wealth of information for the cardiovascular researcher. Among these insights has been the recognition that in cardiac muscle, mitochondria exist in a number of different subcellular locales. This phenomenon has been corroborated in numerous mammalian species including mouse, rat, muskrat, guinea pig, hamster, rabbit, dog, pig, monkey, cow and human (39, 60, 73, 100, 112, 114, 144, 145, 147, 157). This phenomenon is consistent with the evaluation of other non-cardiac cells such as neurons where functional heterogeneity between dendritic, somatic, axonal, and presynaptic segments due to variations in energy demands and calcium (Ca^{2+}) signaling dynamics are associated with structural and biochemical differences in mitochondria situated in a particular neuronal region. Ultimately, this heterogeneity may dictate differences in pathophysiological response of various neuronal regions (89). As with neuronal tissue studies suggest that mitochondrial spatial location within the myocyte may be associated with a particular response to physiological and pathological stimuli (67, 87, 94, 111, 153). With the pioneering development of mitochondrial isolation techniques designed to sequentially fraction spatially-distinct subpopulations of mitochondria utilizing both mechanical and enzymatic procedures (125), efforts to define their individual roles in various pathological conditions has been actively pursued. Using ultrastructure analyses several mitochondrial subpopulations have been identified in the cardiomyocyte, including those residing below the sarcolemma termed subsarcolemmal mitochondria (SSM) and those residing between the myofibril contractile apparatus termed interfibrillar mitochondria (IFM) (Figure 1.1)

(75). Additionally, a specific population of mitochondria, isolated as IFM, resides in the perinuclear region.

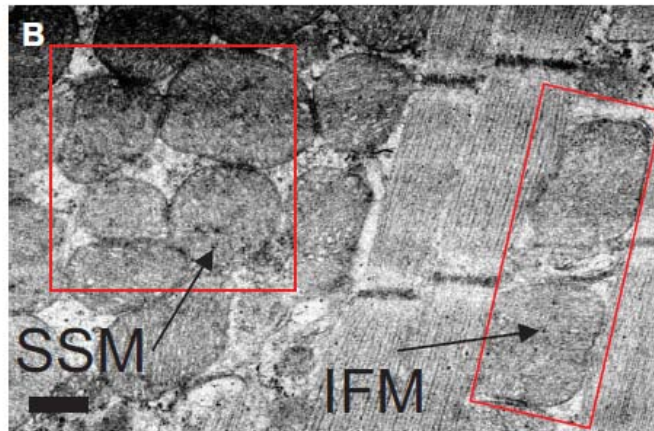


Figure 1.1 Electron micrograph depicting SSM and IFM subpopulations.

1.4a Structural Differences

Structural alterations between mitochondria located in different sub-cellular locales have been reported using a number of diverse experimental techniques. Using thin sections from left ventricular tissue of Japanese Monkeys (*Macaca fuscata*), scanning electron microscopy and transmission electron microscopy revealed distinct populations of mitochondria including perinuclear mitochondria, IFM and SSM (144). Perinuclear mitochondria were clustered at the nuclear poles and mostly spherical in shape with lengths ranging from 0.8-1.4 μm . These mitochondria contained well-developed curved cristae with relatively little matrix area. In addition, IFM were identified and observed in longitudinal rows between the myofibrils, occupying the entire space between Z-lines (54) and bookended by the junctional sarcoplasmic

reticulum (104)(Figure 1B). IFM were elongated in shape with usually one mitochondrion existing per sarcomere. IFM were approximately 1.5-2.0 μm in length and their cristae structures also displayed curved configurations. Finally, SSM were located beneath the sarcolemma (plasma membrane) and were somewhat more variable in length (0.4-3.0 μm), possessing closely packed cristae. Overall, perinuclear mitochondria were smaller than IFM were in size and possessed a more rounded shape (104), while SSM were varied in size and shape displaying oval, spherical, polygonal and horse-shoe patterns (144). Others have reported similar ultrastructural patterns using confocal imaging of HL-1 cells in the perinuclear region, which contain mitochondria clustered around the nucleus (95). En bloc staining of human papillary muscle revealed differences in staining patterns between SSM, IFM and perinuclear mitochondria suggesting differences in chemical properties and metabolic activities (41). These findings are consistent with flow cytometric analyses which employ membrane-dependent dyes (i.e. MitoTracker Deep Red 633) coupled with flow cytometry size calibration microspheres to determine absolute mitochondrial size and internal granulation in both SSM and IFM (37-39, 173). Insight into subcellular mitochondrial distribution has also been afforded by probability density analyses. Using a 3-D modeling approach coupled with MitoTracker Red staining enabling probability density and distribution, isolated rat cardiomyocytes revealed a highly ordered crystal-like pattern in which IFM were arranged in longitudinal rows in clefts between myofibrils (18). In contrast, similar analyses on rainbow trout (*Oncorhynchus mykiss*) cardiomyocytes revealed one single cylinder-shaped layer of myofibrils situated beneath the sarcolemma in which the mitochondrial arrangement was random and chaotic (18). Thus, species differences may be associated with differential mitochondrial spatial patterns in a comparative physiological context.

To gain insight into structural differences in cristae morphology, Riva and colleagues utilized high resolution scanning electron microscopy to examine left ventricular tissue from rat. SSM and IFM displayed distinct morphological patterns in terms of cristae structure with SSM containing primarily (77%) lamelliform cristae which are broad and flat. In contrast, IFM cristae morphology was somewhat mixed and variable with some mitochondria (55%) possessing only tubular cristae while others (24%) possessing some lamelliform cristae mixed with tubular cristae. Further, 21% of IFM possessed only lamelliform cristae. These authors concluded that most of the cristae structure in SSM were lamelliform with occasional tubular cristae, while IFM possessed a reciprocal pattern in which some lamelliform cristae exist despite an abundance of tubular cristae. These authors speculate that the individual cristae morphological patterns may contribute to the functional differences observed between the two subpopulations, including a reduction in intracristal space of tubular cristae. Further, these authors speculate that a reduction of intracristal space could lead to a higher concentration of protons within the structure and enhance ATP synthase activity, which is consistent with IFM functional differences (134). In addition, biochemical composition of the two cristae structures may differ in terms of lipid or protein contents which could ultimately influence structure (134). Indeed, examination of the sphingolipid pool in cardiac SSM and IFM have revealed differences in the content of ceramide, which was higher in the SSM (115).

1.4b Functional Differences

In conjunction with structural differences between spatially-distinct mitochondrial subpopulations, numerous reports have indicated that SSM and IFM possess distinct functional

differences which may result from their particular subcellular locales. Mitochondria residing in the perinuclear region of the cell have been suggested to generate ATP that drives mitochondrial metabolism close to the nucleus. In contrast, it has been hypothesized that IFM supply ATP for contraction, whereas SSM are involved primarily in the provision of ATP for active transport of electrolytes and metabolites across the sarcolemma (116, 125, 136, 144). Though these postulations have not been definitively determined experimentally, they are consistent with the concept that the spatial location of a given mitochondrion is reflective of the processes in which it supplies ATP and the given locale in which it resides.

1.5 Pathological influence on mitochondrial subpopulations

Mitochondrial subpopulations residing in different sub cellular locale are differentially influenced during pathological insult (75). Ischemia studies in canines show structural alterations in the SSM subpopulations, including decrease in membrane fluidity (145). When examining a rat model of chronic hypoxia (11% oxygen exposure for 14 days), Heather et al. observed effects in both SSM and IFM subpopulations as displayed by decreased state 3 respiration rates (71). Because of its crucial role in cellular processes including ROS formation, mitochondrial dysfunction has been implicated as a central contributor in the development of aged heart (40). Fannin et al. observed decreased oxidative phosphorylation (OXPHOS) rates and cytochrome oxidase enzyme activities in the IFM subpopulations of aged rat model when compared to a young model (53). Myocardial infarction studies in rats subjected to coronary artery ligation treatment reveal decrements in respiration rates, electron transport chain (ETC) complex III protein contents and activities in addition to decreased mitochondrial cytochrome c

levels in both SSM and IFM subpopulations (71). Ischemia and reperfusion (I/R) studies display mitochondrial dysfunction in both subpopulations as evidenced by decreased OXPHOS and ADP/ATP translocase activity in rat models undergoing 20 min of ischemia and 30 minutes of reperfusion (46). Studies examining exercise training and its effects on cardiac mitochondrial subpopulations are sparse. Aged C57BL/6J mice subjected to treadmill training protocol displayed increased IFM hypertrophy and loss of matrix and cristae, as well as formation of giant mitochondria (33). However, Fischer 344 rats subjected to long-term voluntary wheel running coupled with caloric restriction showed a reduction in H₂O₂ production and lower manganese superoxide dismutase (MnSOD) activities in both SSM and IFM subpopulations. Interestingly, these authors observed a significant increase in protein carbonylation in SSM, which was not observed in IFM (88). These authors suggest that the increased protein carbonylation observed in SSM may be related to the decreased MnSOD activity in this subpopulation. In contrast, because IFM possess higher antioxidant enzyme activities, a resultant increase in protein carbonylation was not observed despite also displaying decreased MnSOD activities (88). Numerous groups have investigated the effects of specific drugs on cardiac mitochondrial subpopulations. In a study by Duan et al. investigating the effects of verapamil in rat cardiac mitochondrial subpopulations, the authors report significant reversal of oxidative phosphorylation depression in SSM, which was induced by treatment with phosphate (47).

1.5a Pathological Influence: T1DM

Studies have examined cardiac mitochondrial subpopulations in different metabolic diseases including DM. Cardiovascular complications and heart disease are the leading cause of

morbidity and mortality in patients with DM (63). Diabetic cardiomyopathy, characterized by contractile dysfunction independent of atherosclerosis, is the leading cause of heart failure in patients with DM (76, 141, 182). DM can be characterized by lack of insulin production (T1DM) or resistance to insulin (T2DM).

We have previously shown that IFM subpopulations isolated from Swiss-Webster mice rendered diabetic by multi low-dose STZ injections display decreases in size and internal complexity as well as decrements in ETC complex I and III function. Moreover, superoxide production and oxidative damage to proteins and lipids are significantly higher in the type 1 diabetic IFM (38). Indeed, we have observed decreases in cardiolipin content of type 1 diabetic IFM (36, 38) which was associated with decrements in cardiolipin biosynthetic pathway constituent cardiolipin synthase, as reflected by decreased protein and enzymatic activity in the IFM (36). Further, we have observed enhanced apoptotic propensity in type 1 diabetic IFM which was associated with increased caspase-3 and 9 activities, mPTP opening, Bax and cyclophilin D protein contents, with decrements in mitochondrial cytochrome *c* content and Bcl-2 levels (173). Proteomic approaches utilizing isobaric tags for relative and absolute quantitation and two-dimensional-differential in-gel electrophoresis reveal a greater impact in IFM proteomic make-up during type 1 diabetic insult characterized by a decreased abundance of fatty acid oxidation and electron transport chain proteins. Furthermore, mitochondrial protein import is compromised in type 1 diabetic IFM providing a potential mechanism accounting for proteomic dysregulation associated with the content of nuclear-encoded mitochondrial proteins (9). Overexpression of mPHGPx has been shown to increase ETC complex function, attenuate H₂O₂ production and lipid peroxidation in type 1 diabetic IFM. Moreover, reversal of protein import dysfunction and lessened proteomic loss in type 1 diabetic IFM was observed with preservation

of oxidative phosphorylation, tricarboxylic acid cycle and fatty acid oxidation processes as indicated by Ingenuity Pathway Analysis (7). Slc25a3, an inner membrane protein transporter involved in the provision of inorganic phosphate to the mitochondrial matrix, is decreased specifically in type 1 diabetic IFM, which is associated with decreased ATP synthase activity and ATP production (11). Fancher et al., investigating the effects of type 1 diabetes mellitus on the function and expression of ATP-dependent K^+ channels in FVB mice, observed a decrease in pore forming subunit Kir6.1 in both subpopulations whereas diazoxide-sensitive sulphonylurea receptor SUR1 was only decreased in IFM (50). In summary, the majority of studies indicate that type 1 diabetes mellitus primarily affects the IFM. It is unclear as to why this phenomenon occurs but may be related to higher respiration rates, membrane potential and inherent protein import rates in the IFM subpopulation.

1.6 Mitochondrial dysfunction

Mitochondrial dysfunction has been shown to be central to the pathogenesis of T1DM. Cardiac abnormalities observed in diabetic cardiomyopathy has been related to mitochondrial dysfunction including but not limited to enhanced oxidative stress, improper calcium handling, and mitochondrial morphology and number.

1.6a Mitochondrial dysfunction and oxidative stress

Mitochondrial respiratory chain is the major source of ROS production. ROS generation is mainly due to electrons leakage from complexes I and III of the ETC chain thus generating incompletely reduced forms of oxygen (20, 150). Approximately 90% of basal cellular ROS is produced by the mitochondrion in the cardiomyocytes (34). Mitochondrial ROS can cause

cellular damage by oxidation of proteins, conversion of lipids to form lipid peroxidation products. Moreover, ROS can increase intracellular nitration by generating peroxynitrate (158) and are also involved in damage to mitochondrial DNA(166). Increased ROS generation from cardiac mitochondria has been examined in different T1DM animal models (38, 146, 158). Proteomic study examining cardiac mitochondrial proteins in alloxan-induced type 1 diabetic rats provided evidence of tyrosine nitration (158). Further, overexpression of metallothionein, an antioxidant protein have shown reduction in nitrosative damage in STZ-treated mice (28) as well as restoration of normalized levels of oxidized glutathione in OVE26 mouse model (179). Other studies utilizing overexpression of catalase and MnSOD display preservation of myocardial function and mitochondrial morphology (142, 178). Diabetic IFM subpopulations displayed enhanced superoxide as well as nitrotyrosine content and lipid peroxidation in STZ induced T1DM study by our laboratory (38). Overexpression of mPHGPx attenuated H₂O₂ production and lipid peroxidation in the IFM subpopulations of STZ induced T1DM (7).

1.6b Mitochondrial dysfunction and calcium handling

Adequate amount and intricate interactions of Ca²⁺ and ATP is required for cardiomyocyte contraction. Mitochondria aid in restoration and emission of Ca²⁺ to regulate excitation-contraction coupling within the heart (83). In addition to coupling within the heart Ca²⁺ also regulates energy metabolism i.e. ATP production as well as activation of apoptosis through mPTP opening (66). Indeed, in STZ induced T1DM model, lower rates of mitochondrial Ca²⁺ uptake have been observed, which is associated with development of hyperglycemia (56). In another study, although it was observed that calcium uptake was similar

in control versus diabetic animals; diabetic mitochondria were unable to retain the accumulated calcium uptake due to increased opening of the mPTP. This phenomenon was absent in the presence of mPTP inhibitor cyclosporin (123). Similar to these findings, we have observed increased rates of mPTP opening as well as decreased mitochondrial cytochrome c content in IFM subpopulations isolated from STZ treated type 1 diabetic mice (173). Hence, Ca^{2+} handling is impaired in diabetes thus resulting in contractile abnormalities, compromised energy metabolism as well as enhanced mitochondria derived cellular apoptosis.

1.6c Mitochondrial dysfunction and mitochondrial morphology

Alterations of mitochondrial morphology have been observed in T1DM. Examination of skeletal muscle mitochondria from STZ induced diabetic rats showed a loss of cristae when compared to control rats (31). Morphological alterations including mitochondrial cristae structure, number, membranes have been examined in different models of type 1 DM. A study by marinara et al. examining alloxan induced diabetic rats revealed mitochondrial swelling, disrupted membranes and cristae structure in addition to decrements in mitochondrial number in heart and liver (108). Further, Shen et al. upon examination of OVE26 mouse model of type 1 diabetes observed increased focal regions with severe mitochondrial damage in diabetic hearts (143). A more recent study utilizing electron microscopy to examine mitochondrial structure in akita mouse model of type 1 diabetes revealed decrements in mitochondrial cristae density in cardiac mitochondria (25). Another study by the same group showed gross mitochondrial structure accompanied by reduced cristae density in cardiac mitochondria (24). Interestingly, studies by our laboratory have shown decreases in size and internal complexity of IFM

subpopulations isolated from STZ induced type 1 diabetes mouse model (38). Hence, studies examining mitochondrial structure of type 1 diabetes mouse models show gross mitochondrial structure including reduced cristae structure and density suggesting structural damage to the inner mitochondrial membrane.

1.7 Inner Mitochondrial Membrane

The inner mitochondrial membrane (IMM) houses the ETC complexes, OXPHOS machinery, metabolite carriers and enzymes of mitochondrial metabolism. IMM and the proteins residing in it are thus particularly susceptible to ROS damage.

1.7a IMM sub domains

The IMM is composed of two morphologically distinct sub domains, the inner boundary membrane and the cristae membrane (107, 186). These two subdomains have been indicated as distinct compartments of the inner mitochondria, largely due to their asymmetric protein distribution (164, 175, 186). The inner boundary membrane is in close proximity to the outer membrane whereas the cristae membrane is composed of large tubular invaginations that protrude into the matrix space and is the major site for ATP production by OXPHOS (65, 164, 175). Morphological characterization by electron microscopy reveal that the region between the inner boundary membrane and cristae membrane consist of narrow tubular openings with a distinct diameter, length and high membrane curvature known as crista junctions (107, 128, 133, 186). A single section through 3D tomogram of mitochondrion depicts structural features along with the dimensions of the mitochondria (57) (Figure 1.2). A key protein residing in the inner

mitochondrial membrane is structural protein mitofilin, which has been well established for its role in cristae morphology (86).

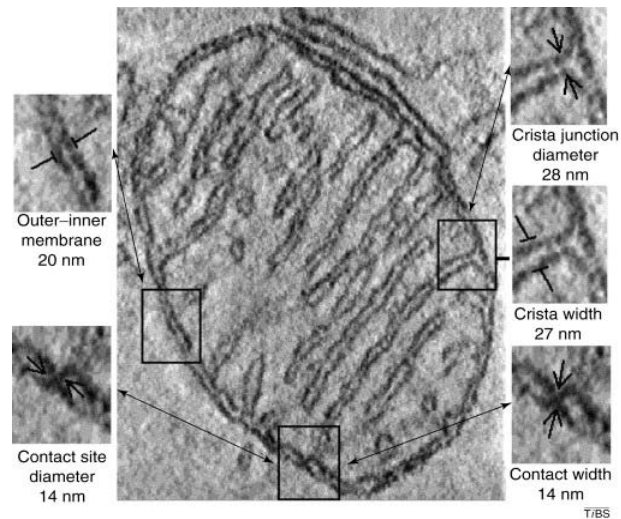


Figure 1.2 3D tomogram of the mitochondrion (57).

1.8 Mitofilin

One of the key findings towards understanding the IMM architecture along with cristae morphology was the identification of mitofilin. Because of its abundance in the human heart it was originally termed as heart muscle protein by Icho et al. (82). Later studies identified mitofilin as an integral membrane protein anchored in the inner mitochondrial membrane with an amino-terminal transmembrane domain (64, 122). It is localized in the cristae junction (90) and plays a role in maintaining cristae morphology (86). Deletion of mitofilin results in altered cristae morphology, loss of cristae junctions as well as mitochondrial DNA (86, 128). Moreover,

mitofilin is a critical component of a large multi-protein complex known as mitochondrial contact site and cristae organizing system (MICOS) (127), which functions as a central organizer of mitochondrial architecture, cristae junctions and determines cristae morphology. MICOS is comprised of Mio10, Aim5, CHCHD3, MOMA-1 and mitofilin (4, 69, 77, 165). Interactions of mitofilin with outer and inner membrane proteins are crucial for mitochondrial function, protein biogenesis, and membrane organization (4, 25, 42, 90, 165, 176, 184). Different interaction complexes of mitofilin have been reported (77, 165, 169, 184). A decrease in mitofilin level has been observed in many human diseases such as Down syndrome (15, 117), Parkinson's disease (26, 160, 161), cancer (25, 106) and diabetic cardiomyopathy (10). Proteomic studies from our laboratory have shown a significant decrease of mitofilin content in IFM subpopulations isolated from STZ induced type 1 diabetic mice (9). Interaction of mitofilin with other proteins in MICOS complex is crucial for mitochondrial morphology and function. With downregulation of mitofilin in IFM of type 1 diabetes mellitus, studies investigating mitofilin and its potential interaction with other novel proteins, as well as over expression of mitofilin in restoring mitochondrial structure and function are crucial to understand the pathological manifestations of type 1 diabetes mellitus.

1.9 Micro-RNA (miRNA)

miRNAs are endogeneous 22-23nt RNAs that play a significant role in gene expression in animals and plants by targeting mRNAs of protein-coding genes for posttranscriptional regulation or translational repression. miRNAs are critical to cell physiology, development and have been implicated in pathological processes such as autoimmune diseases, viral infections,

cancer and diabetes (11, 80, 92, 124, 126, 180). miRNAs were first identified during larval development of *C. elegans* in 1993 (97).

The biogenesis of matured miRNA initiates in the nucleus, with RNA Polymerase II transcribing miRNAs to produce pri-miRNA transcript that has a hairpin loop, capped 5' end and polyadenylated tail which can be thousands of base pairs in length. The nuclear cleavage of pri-miRNA by Drosha RNAse III endonuclease (98) liberates a 60-70nt stem loop intermediate known as pre-miRNA (99, 183). This pre-miRNA is then actively transported from the cytoplasm to the nucleus by Ran-GTP and export receptor Exportin-5 (105, 181). Nuclear cut by Drosha identifies one matured end of the miRNA whereas the other end is processed by the RNAse III endonuclease cytoplasm enzyme called Dicer (98). Dicer first recognizes the double stranded portion of the pre-miRNAs and at about two helical turns away from the base of the stem loop, it cuts both strands of the duplex. This produces an imperfect duplex that comprises the mature miRNA and similar sized fragment derived from opposing arm of the pre-miRNA. The final stage of miRNA biogenesis involves the formation of RNA induced silencing complex (RISC). Dicer unravels the miRNA from its complementary sequence and leaves the single stranded functional miRNA. Then, along with cofactors transactivating response RNA binding protein and other protein activator of interferon induced protein kinase, Dicer helps to bring miRNA to a member of the argonuate family (AGO), thus forming the RISC complex (131). When bound to the mRNA 3' untranslated region, AGO proteins functions in miRNA repression by the inhibition of protein synthesis (55). After the formation of RISC, miRNAs particularly target AGO in RISC, to a specific mRNA for binding and subsequent inhibition. Thus, assembly of RISC and binding of miRNA with mRNA in the presence of AGO proteins are mandatory for miRNA regulated repression. As for the binding of miRNA to the mRNA there is a presence of

2-7 nt seeding region. Once bound to this seeding region, miRNAs can cause translational repression, deadenylation, endonucleolytic cleavage ultimately causing mRNA degradation (103). The figure below represents the biogenesis of miRNA with the RISC component (6).

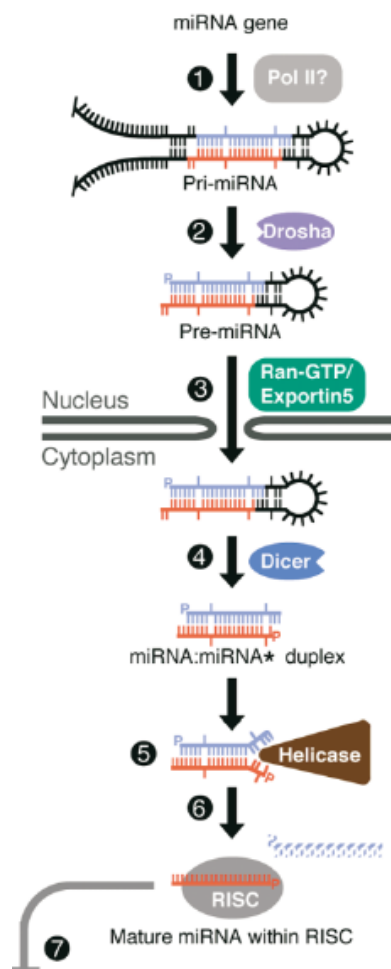


Figure 1.3 Biogenesis of a miRNA (6).

1.9a miRNAs in physiology

miRNAs have been found to regulate cellular functions including but not limited to cell growth, proliferation, differentiation and apoptosis. Because of their role in differentiation, miRNAs are thought to play role in specifying tissue identity and thus expression profile of miRNAs in a specific cell type can be used as a marker for cell type identification (185). Furthermore, miRNAs regulating cell differentiation required for tooth patterning, size, and shape and number destination have been identified (30). miRNAs have been investigated for their potential role in controlling the fate and behavior of stem cells. A study by Gangaraju et al. showed that miRNA-296 promoted embryonic cell differentiation whereas miRNA-22 inhibited the process. In addition to this, they showed that self-renewal of stem cells is promoted by the miR-290-295 cluster which has been shown to silence OCT4 (61). Similarly, studies examining miR-26a and miRNA-138 show that they modulate osteogenic differentiation of stem cells by targeting the SMAD1 transcription factor (49, 61). Hence, miRNAs have been examined for their role in physiology and development.

1.9b miRNAs in pathologies

Involvement of miRNAs in human pathologies were first identified in humans with mutations in fragile (a RISC cofactor) or DGCR 8 (a Drosha cofactor) suffering from mental retardation and DiGeorge syndrome respectively. Almost 50 % of human miRNA genes have been found in genetic loci that have been associated in cancers (14). Upon examination of miRNAs profile in head and neck squamous cell carcinomas (HNSCCs), miRNA-451 was identified as a potential prognostic marker with miRNAs 375 and 106b-25 clusters shown to

mediate development and progression of HNSCC (80). Furthermore, a study by Henson et al. found that downregulation of miR-100 and miR-125b are observed in oral squamous cell carcinoma and could be the potential reasoning behind low sensitivity to ionizing radiation (72). With advent in technologies, increased miRNA profiling in pathologies are being investigated. Characterization of miRNAs expression changes in acute cardioprotection by ischemic pre and post conditioning in rat hearts revealed a total of 18 miRNAs altered by pre and post conditioning or both (163). A similar study by Welten et al. investigating inhibition of miRNAs 329, 487b, 494, and 495 showed increased neovascularization and blood flow recovery after ischemia (170). Hence, a lot of studies have reported altered miRNA profiles as well as role of specific miRNAs in different pathologies.

1.9c miRNAs within the heart

miRNA expression analyses through microarray have demonstrated distinct cellular and tissue specific expression patterns of multiple miRNAs (12, 102). Although most miRNAs are ubiquitously expressed, several miRNAs are enriched within the heart. miRNAs 208a and 208b are cardiac specific miRNAs that are co-expressed, have identical seed sequences and are suggested to regulate same set of genes at different stages of development (29, 162). In addition to these, miRNAs 1, 133, 499 are also highly expressed in the heart (16). Because of spatial presence of specific miRNAs in the heart, role of miRNAs in regulation of cardiac functions including cardiomyocyte differentiation, cardiac development and ventricular hypertrophy have been elaborately studied (5). Furthermore, structural characteristics and mechanisms of miRNAs

have been studied to examine their effects upon development of heart failure, hypertrophy as well as treatment of heart failure (177).

High density microarrays analyses of human atrial tissue in patients with atrial fibrillation undergoing cardiac surgery revealed an elevated expression of miRNA-21. The authors show that expression of miRNA-21 is related to atrial fibrosis and might affect its occurrence and can be used as a biomarker for cardiac surgery management (120). Similarly, an analysis of global expression of miRNAs in physiological left ventricular hypertrophy model revealed a set of miRNAs (-26b, -150, -27a, -143) that can potentially modulate hypertrophy (109). A study by Huang et al. investigating miRNA-34a provided evidence that miRNA-34a plays a critical role in progression of cardiac fibrosis through its targeting of Smad4. Further, the authors conclude that inhibition of miRNA-34a could potentially be a promising strategy in the treatment of cardiac fibrosis (79). Similarly, study examining miRNA-30d revealed its role in regulating cardiomyocyte pyroptosis by targeting foxo3a in diabetic cardiomyopathy. The authors suggest that miRNA-30d could be a promising therapeutic target for management of diabetic cardiomyopathy (101). In conclusion, an array of miRNAs in the heart has been studied for their potential role as therapeutic target and strategy for the treatment of many cardiac diseases.

1.9d miRNAs within the mitochondrion

Mitochondrion plays a critical role in many cellular processes, metabolic pathways which require and utilize proteins encoded from both nuclear and mitochondrial genome. Regulations of proteins in the mitochondria are crucial for its proper functioning. An appropriate balance of mRNA and protein levels is essential for cell survival. Presence of miRNAs in the mitochondria

and their potential roles in regulation of mRNA and proteins are being investigated in both physiological and pathological conditions (149). Recently, miRNAs have been found to be present in mouse and rat liver mitochondria and their roles have been associated with likely regulation of apoptosis, cell proliferation, and differentiation (17, 93). A study by Das et al. examining translocations of nuclear miRNAs into the mitochondria revealed that miRNA-181c was indeed present in the mitochondria and regulated mitochondrial function by targeting cytochrome c oxidase subunit 1 (mt-COX1) mRNA of the mitochondrial genome (43). Further, a study by Sripada et al. utilized deep sequencing approach to characterize the presence of miRNAs in pure fractions of mitochondria isolated from HEK293 and HeLa cells. 428 known and 196 putative novel miRNAs were present in mitochondria of HEK293 cells with 327 known and 13 novel in HeLa cells. Additional analyses of the identified miRNAs showed their potential association with critical cellular processes like RNA turnover, apoptosis, cell cycle and nucleotide metabolism (148). Taken together these findings suggest that further investigation of miRNAs in the mitochondria could potentially help us understand mitochondrial dynamics as well as the role of miRNA in mitochondrial dysfunction. Moreover, presence of differential profile of miRNAs in mitochondrial subpopulations could lend insight upon structural and functional differences observed within spatially distinct SSM and IFM mitochondria.

1.10 Summary

Almost 40 % of populations over 20 years of age in the United States are hyperglycemic. As of 2012, 29.1 million Americans were suffering from diabetes and the total direct and indirect cost of the disease was at a staggering 245 billion dollars. Research endeavors geared towards

understanding the etiology and mechanism of DM is absolutely necessary for better diagnoses as well as therapeutic developments. Morphological alterations of the mitochondrial structure as well as regulation of the mitochondrial genome by miRNAs are associated with mitochondrial dysfunction and diabetic cardiomyopathy. Hence, the goal of this dissertation was to examine the role of mitochondrial structural as well as alterations in mitochondrial miRNA profile in the development of diabetic cardiomyopathy during type 1 DM. Further, a transgenic mouse model overexpressing mitofilin, an inner mitochondrial membrane structural protein was utilized to examine the beneficial effects of mitochondrial structural restoration during type 1 DM.

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Chapter 2:

Novel association of mitofilin with ATP synthase subunit b in mitochondrial subpopulations

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Abstract

Mitofilin is an inner mitochondrial membrane structural protein, well established for its role in maintaining cristae morphology and structure. It is a central component of the mitochondrial contact site and cristae organizing system (MICOS) complex. Interactions of mitofilin with outer and inner membrane proteins have been reported to be crucial for mitochondrial membrane organization, cristae integrity and inner membrane architecture. Moreover, MICOS has been shown to function in concert with ATP synthase dimers. However, association of mitofilin with ATP synthase subunits is not known. The goal of this study was to investigate known and potential interactions of mitofilin with ATP synthase subunits in cardiac mitochondrial subpopulations during a type 1 diabetic insult. Cardiac mitochondria are comprised of two spatially located mitochondria, subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). Using a gel based technique, mitochondrial proteins immunoprecipitated with mitofilin were subjected to LC-ESI-MS analysis. Proteins from all electron transport chain complexes, structural proteins and proteins involved in protein import were identified in an immunoprecipitated complex. Association of mitofilin with F_0 -ATP synthase subunit b (ATP5F1) was decreased in the diabetic IFM when compared with control. Moreover, interaction of mitofilin with coiled-coil-helix coiled-coil-helix domain 3 (CHCHD3) was trending towards decrements in diabetic IFM. Together these results suggest that mitofilin associates with ATP5F1 which is decreased in type 1 diabetic IFM potentially leading to morphological and ATP synthase dysfunction observed during diabetes.

Keywords

mitofilin, diabetes mellitus, mitochondria, CHCHD3, ATP5F1

Introduction

Mitochondria are comprised of two membranes, the outer membrane and the inner membrane. The inner membrane of the mitochondria further constitutes an inner boundary membrane and cristae membrane (22, 40). The inner mitochondrial membrane houses the proteins required for oxidative phosphorylation, electron transport chain complexes, metabolite carriers, and metabolic enzymes required for metabolism and are hence indispensable for proper mitochondrial functioning (13, 15, 36). Prior findings have suggested that proper integrity of mitochondrial cristae morphology is crucial for mitochondrial structure and function (12, 17, 23, 26).

Recently, various independent studies have led to the identification of a large inner membrane protein complex which is critical for inner membrane architecture, mitochondrial function, cristae organization, formation of contact sites as well as protein biogenesis (1, 5, 16, 18, 33, 34, 38). This protein complex has been named mitochondrial contact site and cristae organizing system (MICOS) (28). Mitofilin, an inner membrane structural protein, has been identified as a critical component of the MICOS complex where it interacts with Mio10, Aim5, CHCHD3 and MOMA-1 to form the complex (39). The role of mitofilin in regulation of cristae morphology, mitochondrial membrane organization and formation of crista junctions has been well established (19, 20, 34). Further, interactions of mitofilin with Sam50 and CHCHD3 to form the mitochondrial intermembrane space bridging (MIB) complex for biogenesis of respiratory complexes (24), metaxins 1 and 2, Sam50, CHCHD3, CHCHD6 and DnaJC11 with a potential role in protein import (37) have been reported. Hence, interactions of mitofilin with both inner and outer membrane proteins have been studied in order to properly understand its role in mitochondrial structure and function.

Examination of cardiac mitochondria is complicated by the presence of two spatially distinct mitochondrial subpopulations; subsarcolemmal mitochondria (SSM) located beneath the sarcolemma and interfibrillar mitochondria (IFM) situated between the myofibrils. Mitochondrial subpopulation specific interactions of mitofilin have not been studied yet. Hence, the goal of this study was to identify novel association of mitofilin in mitochondrial subpopulations and examine whether the interaction of mitofilin with the identified proteins are altered during a type 1 diabetic insult.

Materials and Methods

Experimental animals and induction of diabetes

The animal experiments in this study conformed to the National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals* and were approved by the West Virginia University Animal Care and Use Committee. Male FVB mice were housed in the West Virginia University Health Sciences Center animal facility on a 12-h light/dark cycle in a temperature controlled room. Mice were given unlimited access to a standard rodent diet and water. Type 1 diabetes mellitus was induced in 6 week old mice following the protocol of the Animal Models of Diabetic Complications Consortium utilizing multiple low-dose streptozotocin (STZ; Sigma, St. Louis, MO) injections as previously described by our laboratory (2-4, 7, 10, 35). Briefly, sodium citrate buffer (pH 4.5) with 50 mg/kg body weight STZ was administered to mice for 5 consecutive days via intraperitoneal injections after 6 hours of fasting. Vehicle control animals were injected with same volume per body weight sodium citrate buffer. Three days following the last injection, hyperglycemia was measured and confirmed (Contour Blood Glucose Test Strips, Bayer Healthcare, Mishawaka, IN). Mice with blood glucose levels greater than 250 mg/dL were considered diabetic. All of the mice injected with STZ became diabetic (values in mg/dL; diabetic 401 ± 81). After diabetic induction, animals were maintained for 5 weeks and then euthanized for further experimentation.

Mitochondrial subpopulation isolation

At 5 weeks post-hyperglycemia onset, FVB diabetic and their littermate control mice were euthanized and their hearts excised. Hearts were rinsed in phosphate buffered saline (PBS, pH 7.4), then blotted dry. SSM and IFM subpopulations were isolated as previously described following the methods of Palmer *et al.* (25) with minor modifications by our laboratory (2-4, 7-11, 35). Mitochondrial pellets were either resuspended in KME buffer (100 mM KCl, 50 mM MOPS, and 0.5 mM EDTA, pH 7.4) for western blot analysis and activity measurements, or in 1.0 % NP-40 wash/binding buffer (100 mM HEPES (pH 7.4), 1.0 % NP-40, 150 mM NaCl) for mitofilin/protein associations. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (6).

Western blot analyses

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was run on 4-12% gradient gels as previously described (21, 35) with equal amounts of protein loaded. Relative amounts of mitofilin, cytochrome *c* oxidase (COX IV), ATP5F1, ATP5A, and CHCHD3 were assessed using the following primary antibodies; anti-mitofilin rabbit antibody (product no. ab48139, Abcam, Cambridge, MA), anti-COX IV rabbit antibody-mitochondrial loading control (product no. ab16056, Abcam, Cambridge, MA), anti-ATP5F1 mouse antibody (product no. ab117991, Abcam, Cambridge, MA), anti-ATP5A mouse antibody (product no. ab110273, Abcam, Cambridge, MA), and anti-CHCHD3 rabbit antibody (product no. ab98975, Abcam, Cambridge, MA). The secondary antibodies used in the analyses were goat anti-rabbit IgG horseradish peroxidase conjugate (product no. 10004301, Cayman Chemical Company, Ann Arbor, MI) for mitofilin, COX IV, and CHCHD3, and goat-anti mouse conjugate (product no. 31430; Pierce

Biotech, Rockford, IL) was used for ATP5F1, and ATP5A. Detection of signal was performed using a Pierce ECL Western blotting substrate detection system according to the manufacturer's directions (Thermo Fisher Scientific Inc., Rockford, IL). Quantification of chemiluminescent signals were assessed using a G:Box Bioimaging System (Syngene, Frederick, MD), and data were expressed as arbitrary optical density units. Densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD). Protein loading was confirmed using COX IV.

Immunoprecipitation analyses

Dynabeads[®] protein G (product no 10003D, Life technologies, Grand Island, NY) were used to determine protein associations with mitofilin per the manufacturer's instructions. Briefly, 100ug of mitochondrial protein resuspended in 1.0 % NP-40 wash/binding buffer was incubated with mitofilin antibody overnight. 40ul of protein G beads were then added to the tubes and rocked at 4°C for an hour. The antibody binds to the Dynabeads via their Fc-region. The tubes were placed in a magnet and beads were washed three times with the wash/binding buffer. The supernatant was removed by aspiration and the magnetic beads were eluted with 4X Laemmli sample buffer and heated to 70°C. Western blots were performed on the eluted protein and probed with protein of interest so as to examine the association with mitofilin.

Protein identification by LC-ESI-MS/MS

In order to identify proteins associated with mitofilin, 1000ug of mitochondrial protein was used for immunoprecipitation as described above. After elution of magnetic beads with 4X Laemmli sample buffer, it was sent to Protea Biosciences, Inc (Morgantown, WV) for protein identification by LC-ESI-MS/MS. Briefly, samples and molecular weight marker were loaded onto ProteaGel Mini Precast gel and run in 180v for 30 minutes. After electrophoresis, the gel was stained with SYPRO Ruby red stain for overnight and imaged at 550-750 nm. Varying molecular weight regions were cut and soaked in 150mM ammonium bicarbonate (AmBic) and then acetonitrile. The samples were then dried down on a lyophilizer and treated with 10mM dithiothreitol (DTT) in 50mM AmBic followed by an hour incubation at 56°C. After a 30 second centrifuge at 4000 RPM, excess DTT was removed and 50ul of 55mM iodoacetamide (IA) in 50mM AmBic was added to the gels and incubated in the dark for an hour. After drying down the samples, trypsin in 50mM AmBic was added and incubated at 37°C overnight. Gel pieces after centrifugation were lyophilized and reconstituted in 80ul of acetonitrile/water/formic acid for LC-ESI-MS analysis. Liquid chromatography was done using Shimadzu LC-20AD HPLC (Tokyo, Japan) and control software was Analyst 1.5. ESI mass spectrometer was performed using QTrap 5500 (Ab Sciex, Toronto, Canada) and the ionization method used was electrospray ionization in positive ion mode with a voltage at 5.0 kV. Summary of the proteins and peptides identified were presented in the Excel data file.

Ingenuity pathway analysis

After protein identification through mitofilin pull down immunoprecipitation and LC-ESI-MS/MS, the accession numbers and pull down proteins were tabulated and imported into

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) for canonical pathway analysis. The software was utilized for construction of interacting proteins networks associated with mitofilin. IPA contains a database that uses the most current knowledge available on genes, proteins, protein interactions, and metabolic pathways needed for protein network construction.

Blue native page

To assess electron transport chain (ETC) complex V content in control and diabetic mitochondrial subpopulations, blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as previously described (2, 8) with modifications according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) using equal amounts of protein. Briefly, isolated mitochondria were solubilized with 1% digitonin on ice. After addition of Coomassie G-250, samples were run on 4–16% NativePAGE gels. Following BN-PAGE, gels were placed in a fixed solution containing 40% methanol and 10% acetic acid followed by microwaving for 45 seconds at 1,100 watts. Gels were then washed for 15 minutes at room temperature after which the solution was decanted. Destaining was accomplished by addition of 50 ml of an 8% acetic acid solution and microwaved a second time for 45 seconds at 1,100 watts. The gel was then shaken at room temperature until the desired background was obtained. To control for destaining time and enable comparison between gels, each band of interest was expressed per the molecular weight marker 480 kDa band. The gel was then scanned and densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD).

Statistics

Means and SEMs were calculated for all data sets. Data were analyzed using Student's t-test (GraphPad Software Inc., La Jolla, CA). Differences between control and diabetic groups were considered significant when a $P < 0.05$ was observed.

Results

Protein identification by LC-ESI-MS/MS analyses

Immunoprecipitation with mitofilin followed by gel electrophoresis was performed to identify proteins that were pulled down with mitofilin. After gel electrophoresis, the bands were cut in different sizes as shown in Figure 2.1. The band sizes cut were 15kDa, 25kDa, 35kDa, 50-100kDa and 100kDa plus. Further analyses were performed in the cut gels as described in the methods section. Finally, LC-ESI-MS/MS analyses were performed to identify the list of proteins that were pulled down with mitofilin.

Canonical pathways

Using IPA software-fed proteins pull down with mitofilin, we attempted to predict canonical pathways associated with identified proteins within the mitochondrion. Upon analyses of the pathways we identified proteins involved in Electron transport chain complexes I, II, III, IV and V, proteins involved in mitochondrial structure and protein import (Figure 2.2). CHCHD3, pulled down with mitofilin is crucial for maintaining crista integrity and mitochondrial function (12). Further, mitofilin with CHCHD3 and Samm50 form a large protein complex named mitochondrial intermembrane space bridging complex which is crucial for preservation of cristae and in the assembly of respiratory chain complexes (24).

Mitofilin and CHCHD3 interaction

Because of its role in maintaining cristae morphology, we examined association of mitofilin with CHCHD3 in control and diabetic mitochondrial subpopulations (Figure 2.3A). Our laboratory has previously shown decreased mitofilin content in diabetic IFM (3). Further, we

assessed the protein content of CHCHD3 in control and diabetic subpopulations and observed no significant changes (Figures 2.3B-C). We conclude that the observed trending decreased association of mitofilin with CHCHD3 in diabetic IFM is a function of decreased mitofilin content in diabetic IFM.

Novel interaction of mitofilin with ATP5F1

To gain insight into which ATP synthase subunit constituent proteins may be associated with mitofilin, we utilized immunoprecipitation pull down with mitofilin and probed with ATP5F1. Upon examination of mitofilin association with ATP5F1 in control and diabetic mitochondrial subpopulations, we observed a significant decrease in diabetic IFM when compared to control (Figure 2.4A). Further, assessment of ATP5F1 protein content in control and diabetic mitochondrial subpopulations revealed no significant differences in either group (Figures 2.4B-C). We conclude that the significant decrease of ATP5F1 association with mitofilin in diabetic IFM is a function of decreased mitofilin content in type 1 diabetic IFM subpopulations.

Blue native page

To gain insight into whether decreased association of mitofilin with ATP synthase F0 subunit complex results in down-regulation of ATP synthase complex component, we utilized a BN-PAGE approach. This allowed us to evaluate the complex V expression as a whole. Our data indicated no significant differences in complex V content between control and diabetic groups in SSM and IFM (Figures 2.5B-C). We conclude that the decreased association of mitofilin with ATP5F1 in diabetic IFM does not result in decrement of complex V content.

Discussion

Interactions of mitofilin with mitochondrial inner as well as outer membrane proteins have been widely reported. We are the first group to examine known as well as novel interacting partners and associates of mitofilin in mitochondrial subpopulations. No study has examined the differential interaction profile of mitofilin in mitochondrial subpopulation during a type 1 diabetic insult. The following key findings resulted from these studies: 1) Identification of proteins pulled down with mitofilin utilizing LC-ESI-MS/MS analyses revealed proteins from ETC complexes I, II, III, IV and V, protein import machinery in addition to well established structural proteins; 2) Interaction of mitofilin with CHCHD3 is trending towards decreased in type 1 diabetic IFM subpopulations; 3) Novel association of mitofilin with ATP5F1 was observed in mitochondrial subpopulations and this association is decreased in type 1 diabetic IFM when compared with control and; 4) BN-PAGE analyses of Complex V did not reveal any significant changes in protein content in control and diabetic mitochondrial subpopulations. Taken together, these findings provide novel associating partners of mitofilin and reveal decreased interaction of mitofilin in type 1 diabetic IFM. These findings could potentially explain mitochondrial dysfunction observed in type 1 diabetic IFM subpopulations.

Interaction of mitofilin with CHCHD3 and Sam50 have been shown to form MIB which is crucial for cristae preservation and assembly of respiratory complexes (24). Further, others have shown existence and requirement of C-terminal domain of mitofilin for interaction with Tob55/Sam50 complex which could potentially regulate outer membrane protein import and formation of crista junctions (20, 37). Our findings agree with these studies as both CHCHD3 and Sam50 are pulled down with mitofilin. Further, CHCHD3 along with mitofilin are part of MICOS complex which is crucial in regulating cristae morphology and mitochondrial function

(16, 18, 28, 33). Previous studies from our laboratory and others have shown morphological alterations specifically in the IFM subpopulations during type 1 diabetic insult (10, 31). We have also shown down regulation of mitofilin in diabetic IFM subpopulations (3). Our findings in this study revealed a trending decreased association of mitofilin with CHCHD3 in diabetic IFM which could potentially result in morphological alterations observed during diabetes as mentioned above. Further, mitofilin along with CHCHD3 has been shown to cristae morphology and integrity (12, 19, 38).

Proteins which are not a constituent of the MICOS complex have also been associated with regulation of cristae morphology and structure (14, 27). Of particular interest to this study, are findings by Paumard et al. who utilized yeast cells to show the potential role of ATP synthase subunits e or g in regulating cristae morphology (27). Further, Rabl et al. showed that deletions of ATP subunits e or g resulted in reduction of cristae tip numbers and crista junctions diameter enlargement. The authors showed that mitofilin and ATP synthase subunits e or g genetically interacted (29). In our current study we identified ATP synthase subunit proteins pulled down with mitofilin. Novel association of mitofilin with ATP synthase subunit b (ATPF1) was observed in mitochondrial subpopulations. Further, the association was decreased in diabetic IFM when compared to control. Previous findings from our laboratory have shown decreased ATP synthase activity in diabetic IFM when compared to control (2, 10). Hence, novel association of mitofilin with ATPF1 could present a potential mechanism in regulating cristae morphology and decreased association observed in diabetic IFM subpopulations could result in structural alterations and dysfunctional ATP synthase activity as observed in type 1 diabetes mellitus.

ATP synthase also referred to as complex V makes ATP through oxidative phosphorylation. Decreased association of mitofilin with ATP5F1, as well as decreased synthase activity observed during type 1 diabetic insult could result in decrement in complex V. In order to examine this, we utilized BN-PAGE technology to assess the protein content. This technology has been utilized before to analyze membrane complexes and diagnose oxidative phosphorylation defects in mitochondria (8, 30, 32). Analysis of complex V content revealed no significant differences between control and diabetic subpopulations. These findings suggest that although a decreased association of mitofilin with ATP5F1 is observed in diabetic IFM, the total protein content of complex V is not changing. Additional studies examining association of mitofilin with other ATP synthase subunits could lend insight into mechanisms contributing to observed ATP synthase dysfunction during type 1 diabetes mellitus.

In conclusion, we report for the first time, interactions and novel association of mitofilin in mitochondrial subpopulations. Further, interaction of mitofilin with CHCHD3 and decreased association of ATP5F1 was observed in diabetic IFM when compared with control. Also, pulled down with mitofilin were proteins associated with ETC complexes, mitochondrial structure as well as protein import which warrants further investigation of role of mitofilin and its associations with these proteins in regulating these mechanisms.

Conflict of Interest

There are no conflicts of interest to disclose.

Grants

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Figure 2.1

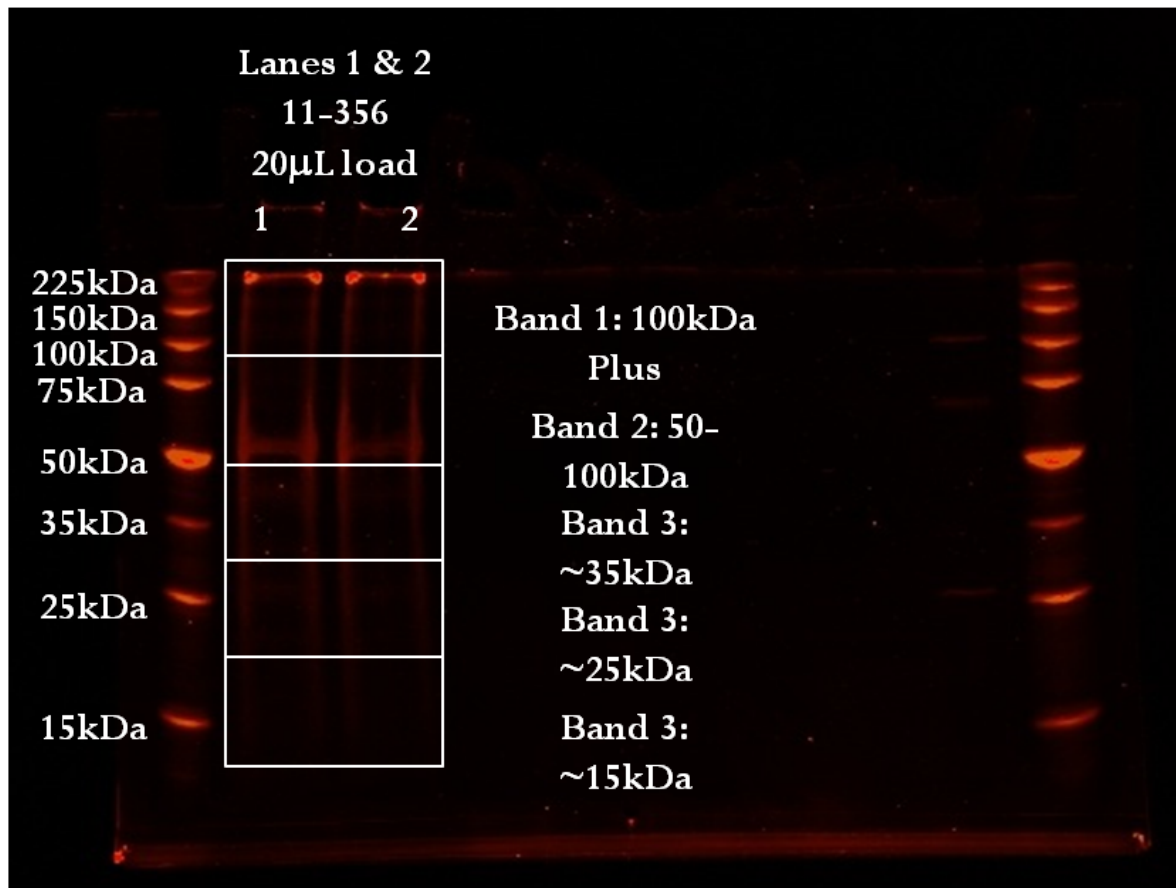


Figure 2.1. Protein identification by gel electrophoresis and LC-ESI -MS/MS. Gel electrophoresis of proteins pull down with mitofilin. Gels were cut in following band sizes: 15kDa, 25kDa, 35kDa, 50-100kDa and 100kDa plus. Each band was then separately run through LC-ESI-MS/MS so as to identify potential protein targets that associated with mitofilin.

Figure 2.2

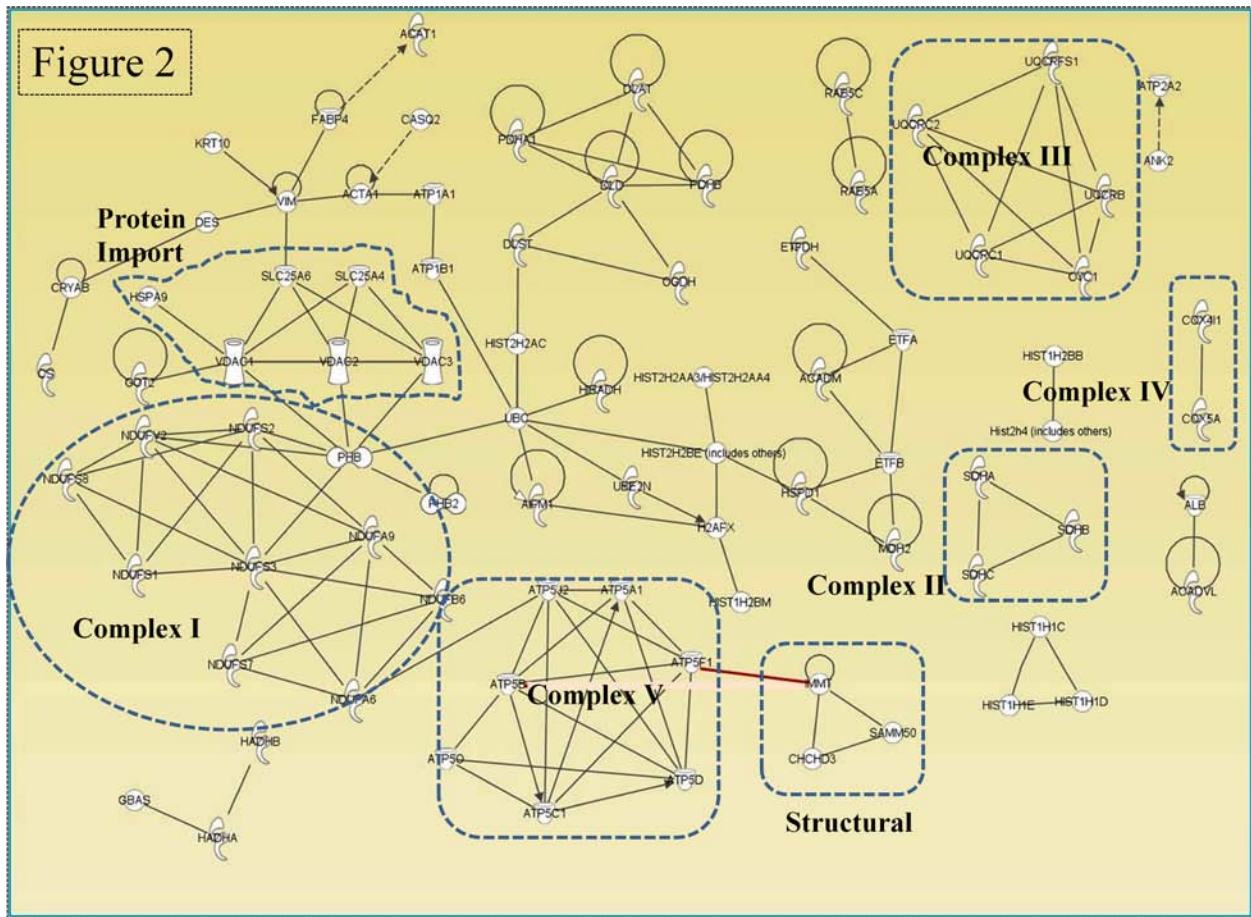


Figure 2.2. Canonical pathways analyses using Ingenuity pathway analysis. Identification of mitochondrial protein networks involving proteins pulled down with mitofilin. Networks involved in ETC complexes, mitochondrial structure, protein import were identified. Red line indicates novel association of mitofilin with ATP5F1 presented in this study.

Figure 2.3

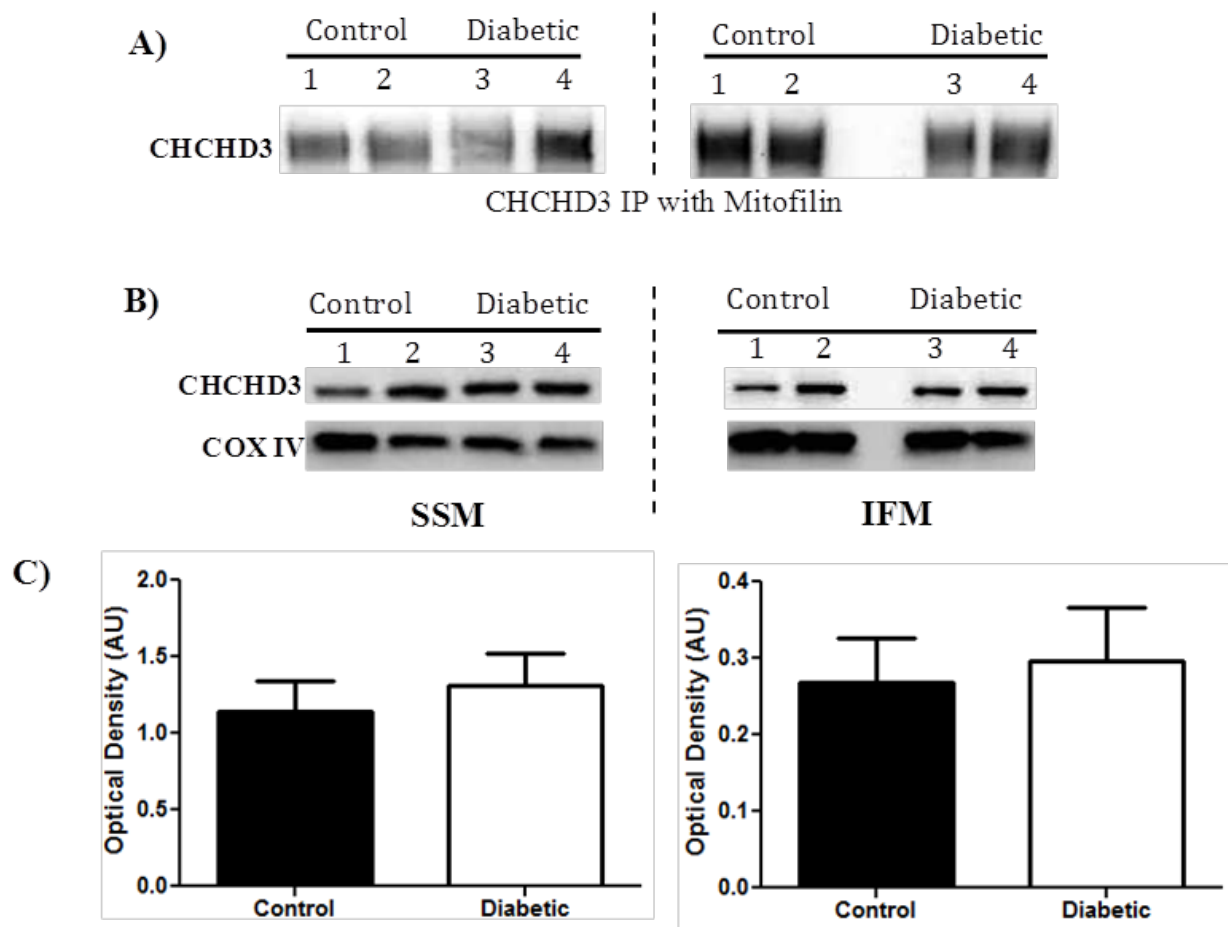


Figure 2.3. CHCHD3 interaction and protein content. A) Interaction of CHCHD3 with mitofilin in control and diabetic mitochondrial subpopulations. Control (lanes 1 and 2) and diabetic (lanes 3 and 4) for both SSM and IFM. B) Western blot analysis of CHCHD3 protein content in control (lanes 1 and 2) and diabetic (lanes 3 and 4) for SSM and IFM subpopulations. Bar graph representation of CHCHD3 protein content over COX IV in C) SSM and IFM subpopulations. Values are presented as means \pm SE. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=4 for each group. SSM: subsarlocemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 2.4

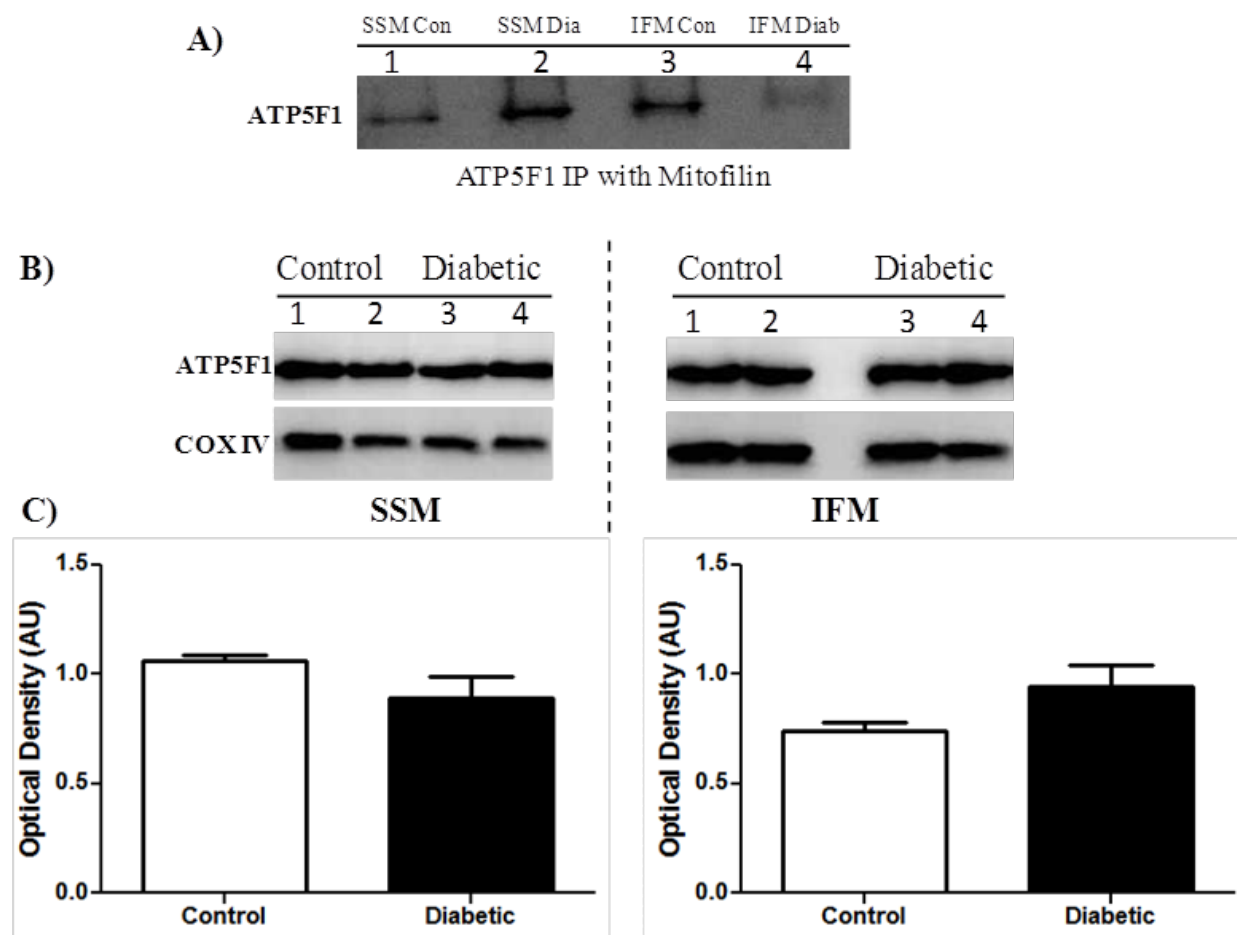


Figure 2.4. ATP5F1 novel association with mitofilin and protein content. A) Association of ATP5F1 with mitofilin in control and diabetic mitochondrial subpopulations. SSM control (lane 1), SSM diabetic (lane 2), IFM control (lane 3), and IFM diabetic (lane 4). B) Western blot analysis of ATP5F1 protein content in control (lanes 1 and 2) and diabetic (lanes 3 and 4) for SSM and IFM subpopulations. Bar graph representation of ATP5F1 protein content over COX IV in C) SSM and IFM subpopulations. Values are presented as means \pm SE. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=4 for each group. SSM: subsarlocemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 2.5

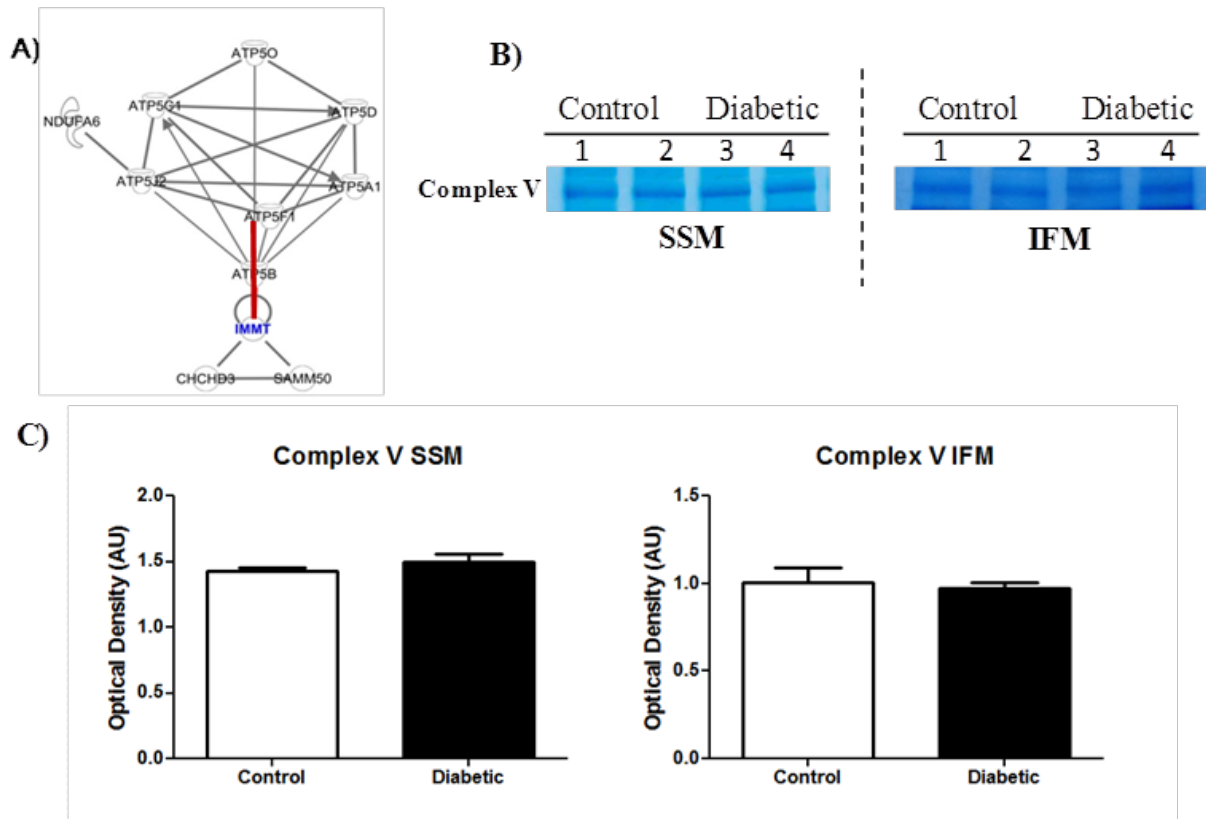


Figure 2.5. BN-PAGE analysis of ETC complex V. A) IPA pathway showing novel association of mitofilin with ATPF1 of ETC complex V. B) ETC complex V expression was examined in control (lanes 1 and 2) and diabetic (lanes 3 and 4) mitochondrial subpopulations using BN-PAGE. C) Bar graph representation of ETC complex V assessed for SSM and IFM control and diabetic groups. Values are expressed as means \pm SE. n=4 for each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Chapter 3:

Transgenic overexpression of mitofilin attenuates diabetes mellitus-associated cardiac and mitochondria dysfunction

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Abstract

Mitofilin, also known as heart muscle protein, is an inner mitochondrial membrane structural protein that plays a central role in maintaining cristae morphology and structure. It is a critical component of the mitochondrial contact site and cristae organizing system (MICOS) complex which is important for mitochondrial architecture and cristae morphology. Our laboratory has previously reported alterations in mitochondrial morphology and proteomic make-up during type 1 diabetes mellitus, with mitofilin being significantly down-regulated in interfibrillar mitochondria (IFM). The goal of this study was to investigate whether overexpression of mitofilin can limit mitochondrial disruption associated with the diabetic heart through restoration of mitochondrial morphology and function. A transgenic mouse line overexpressing mitofilin was generated and mice injected intraperitoneally with streptozotocin using a multi low-dose approach. Five weeks following diabetes mellitus onset, cardiac contractile function was assessed. Restoration of ejection fraction and fractional shortening was observed in mitofilin diabetic mice as compared to wild-type controls ($P < 0.05$ for both). Decrements observed in electron transport chain (ETC) complexes I, III, IV and V activities, state 3 respiration, lipid peroxidation as well as mitochondria membrane potential in type 1 diabetic IFM were restored in mitofilin diabetic mice ($P < 0.05$ for all). Qualitative analyses of electron micrographs revealed restoration of mitochondrial cristae structure in mitofilin diabetic mice as compared to wild-type controls. Furthermore measurement of mitochondrial internal complexity using flow cytometry displayed significant reduction in internal complexity in diabetic IFM which was restored in mitofilin diabetic IFM ($P < 0.05$). Taken together these results

suggest that transgenic overexpression of mitofilin preserves mitochondrial structure, leading to restoration of mitochondrial function and attenuation of cardiac contractile dysfunction in the diabetic heart.

Keywords

mitofilin, diabetes mellitus, mitochondria, electron transport chain

1. Introduction

Mitochondria are ubiquitous, double membranous organelles with an outer membrane and inner membrane. The inner mitochondrial membrane (IMM) of the mitochondria is comprised of an inner boundary membrane which is in close proximity with the outer membrane and cristae membrane [1, 2]. The regions in between the inner boundary and cristae membranes are narrow tubular openings with a fixed diameter and length called crista junctions [1-3]. The cristae membrane is composed of large tubular invaginations that protrude into the matrix space and house the respiratory chain complexes, as well as the F_1F_0 -ATP synthase, rendering it indispensable for proper mitochondrial function [4-7]. Numerous human pathologies have been associated with abnormal mitochondrial structure [8, 9]. Thus, proper integrity of mitochondrial cristae morphology is crucial for mitochondria structure and function.

A number of mitochondrial proteins including optic atrophy 1 (OPA1), coiled-coil-helix coiled-coil-helix domain 3 (CHCHD3), coiled-coil-helix cristae morphology 1 (CHCM1), and ATP synthase subunits have been associated with the regulation of cristae morphology [10-13]. One of the recently identified proteins, mitofilin, has been reported to be requisite for the maintenance of proper cristae morphology. Mitofilin, also known as heart muscle protein [14] due to its high abundance in the heart, is an IMM structural protein specifically localized to the cristae junction [15]. Down-regulation of mitofilin in HeLa cells results in abnormal mitochondrial morphology with concentric layers of inner membrane, reduced cell proliferation, increased apoptosis and elevated reactive oxygen species (ROS) production [16]. Moreover, mitofilin depletion studies in yeast cells and *C. elegans* results in curved and stacked

mitochondrial cristae tubules, increased ROS production, as well as a reduction in cristae junctions which is associated with decreased mitochondrial DNA content [3, 17]. Recently, mitofilin was identified as a critical component of the mitochondrial contact site and cristae organizing system (MICOS) complex where it functions as a central organizer of mitochondrial architecture, cristae junctions and cristae morphology [18-23]. Thus, a number of studies substantiate the significant role of mitofilin in maintaining proper mitochondrial structure and function.

Decrements in mitofilin content have been observed in many human diseases such as Down's syndrome [24, 25], Parkinson's disease [26, 27], Epilepsy [28, 29] and Neurodegeneration [30, 31]. Abnormal mitochondrial morphology, significant reduction in cristae density, as well as decrements in mitofilin content have been reported in the type 1 diabetic heart [32, 33]. Our laboratory has reported that following a type 1 diabetic insult, IFM exhibit greater dysfunction characterized by enhanced oxidative stress, changes in mitochondrial morphology and function as well as altered mitochondrial proteomic signature which is not observed in type 1 diabetic subsarcolemmal mitochondria (SSM) [33, 34].

The IMM proteomic signature is significantly impacted following type 1 diabetic insult and mitofilin is one particular protein that shows significant decrease, specifically in the IFM. The impact of mitofilin loss in type 1 diabetic IFM was associated with changes in mitochondrial morphology and function. Mitofilin overexpression studies in yeast cells have revealed increased diameter as well as branching of cristae and cristae junctions [3]. Nevertheless, overexpression of mitofilin in the context of the diabetic heart have not been undertaken. To address this gap in knowledge, we generated a novel transgenic mouse model of mitofilin overexpression. The goal of the current study was to determine whether mitofilin overexpression provides cardioprotective

benefits to the type 1 diabetic heart and if these effects are associated with improved mitochondrial structure and function.

2. Materials and Methods

2.1 Mitofilin Transgenic Mouse Development

The animal models used in this study conform to the NIH guidelines for the care and use of laboratory animals and were approved by the West Virginia University, School of Medicine Animal Care and Use Committee. Mitofilin transgenic mouse lines were generated by inserting a cDNA encoding the human mitofilin gene into the pCAGGS vector as previously described [35-38]. Briefly, the pCAGGS vector places the mitofilin gene (RG 201854, Origene) under the control of the human cytomegalovirus (CMV) immediate early enhancer and chicken β -actin (Ch. β -actin) promoter with first intron. The mitofilin cDNA was inserted into the XhoI cloning site of pCAGGS via sticky-end ligation (XhoI/XhoI) and blunt-end ligation of an Sgf1/XbaI fragment of approximately 2227 bp (Figure 3.1). The chimeric transgene was cut out of the plasmid by SspI and BamHI digestion, purified, and used to generate transgenic mice. The construct was given to the West Virginia University Transgenic Animal Core Facility where the pronucleus of fertilized eggs from superovulated FVB female mice crossed with FVB male mice, was injected with 1-2 μ l of purified DNA fragment at a concentration of 2 μ g/ml, and transferred into the oviducts of pseudopregnant CD-1 mice. All control and transgenic mice were generated using an FVB background, and experimental procedures were initiated on animals of approximately 5 weeks of age. Animals were maintained in individual microisolator cages within the West Virginia University Transgenic Barrier Facility and given food and water ad libitum.

2.2 Mitofilin transgenic mouse screening

Mouse litters were delivered after 19-20 days of gestation. To verify whether the chimeric transgene was present in the genome, DNA from 3-week-old mice was isolated from tail clips using a Qiagen DNeasy tissue kit (Qiagen, Valencia, CA). Transgene screening was performed by qPCR using a mitofilin probe (#hs00272794-m1; Applied Biosystems, Foster City, CA). Using this approach, only DNA that contains the exogenous mitofilin cDNA was detected. Briefly, isolated tail DNA, probe, and universal master mix were brought up to 25µl and qPCR was performed in a 96 well plate using an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies, Grand Island, NY). A reaction time versus cycle number amplification plot was generated and transgene positive and negative animals were determined.

2.3 Diabetes induction

Male mice were separated into four groups: 1) control; 2) diabetic; 3) mitofilin overexpression; and 4) mitofilin overexpression diabetic. Type 1 diabetes mellitus was induced in diabetic and mitofilin overexpression diabetic mice following the protocol of the Animal Models of Diabetic Complications Consortium utilizing multiple low-dose streptozotocin (STZ; Sigma, St. Louis, MO) injections as previously described by our laboratory [33, 34, 39-42]. Briefly, sodium citrate buffer (pH 4.5) with 50 mg/kg body weight STZ was administered to mice for 5 consecutive days via intraperitoneal injections after 6 hours of fasting. Vehicle control animals were injected with same volume per body weight sodium citrate buffer. Three days following the last injection, hyperglycemia was measured and confirmed (Contour Blood Glucose Test Strips, Bayer Healthcare, Mishawaka, IN). Mice with blood glucose levels greater

than 250 mg/dL were considered diabetic. All of the mice injected with STZ became diabetic and no differences in degree of hyperglycemia was evident with mitofilin transgene presence (values in mg/dL; control diabetic 401 ± 81 , mitofilin diabetic 392 ± 91). After diabetic induction, animals were maintained for 5 weeks and then euthanized for further experimentation.

2.4 Cardiac contractile function

Transthoracic echocardiography was performed as previously described by our laboratory [39] and others [43]. Briefly, mice were anesthetized with inhalant isoflurane and transferred to dorsal recumbency. Using the Vevo 2100 Imaging System (Visual Sonics, Toronto, Canada) and a 32- to 55-MHz linear array transducer, micro-ultrasound images were acquired. M-mode images were captured via the parasternal short axis at midpapillary level with all images acquired at the highest possible frame rate (233-401 frames/s). Left ventricular M-mode images provided end-diastolic and end-systolic diameters and volumes, stroke volume, ejection fraction, fractional shortening, heart rate and cardiac output measurements. All echocardiographic measurements were performed in conjunction with the West Virginia University Animal Models of Imaging Core Facility.

2.5 Human patient population

The West Virginia University Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC) approved all protocols. Individuals undergoing coronary artery bypass graft surgery or cardiac valve replacement at Ruby Memorial Hospital in Morgantown,

West Virginia, consented to the release of their cardiac tissue to the West Virginia University School of Medicine. Consenting individuals were then characterized as non-type 1 diabetic and type 1 diabetic based upon previous diagnosis of diabetes mellitus.

2.6 Preparation of individual mitochondrial subpopulations

At 5 weeks post-hyperglycemia onset, control, diabetic, mitofilin overexpression, and mitofilin overexpression diabetic mice were euthanized and their hearts excised. For human samples, right atrial appendages were removed from patients, pericardial fat was trimmed and the heart tissue was weighed. For both mouse and human samples, hearts were rinsed in PBS (pH 7.4) and SSM and IFM subpopulations were isolated as previously described following the methods of Palmer *et al.* [44] with minor modifications by our laboratory [33, 34, 38-42, 45, 46]. Mitochondrial pellets were resuspended in KME buffer (pH 7.4) for mitochondrial respiration analyses, flow cytometric analyses and enzymatic activity measurements. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard [47].

2.7 Mitochondrial respiration rates

State 3 and state 4 respiration rates were assessed in isolated mitochondrial subpopulations as previously described [48-50] with slight modifications [38, 45]. Briefly, isolated mitochondria were resuspended in KME buffer and protein content was determined by the Bradford method as above [47]. Mitochondria protein was added to respiration buffer and loaded into a respiration chamber which was connected to an oxygen probe (OX1LP-1mL

Dissolved Oxygen Package, Qubit System, Kingston, ON, Canada). The substrates glutamate (5mM) plus malate (5mM) were used to initiate respiration, and measurements of state 3 (250mM ADP) and state 4 (ADP-limited) respiration were made. Values were expressed as nmol of oxygen consumed/min/mg protein.

2.8 ETC complex activities

ETC complexes I, III and IV activities were measured spectrophotometrically as previously described [34, 38, 46, 51]. Briefly, complex I activity was determined by measuring the oxidation of NADH at 340 nm. Complex III activity was determined by measuring the reduction of cytochrome *c* at 550 nm in the presence of reduced decylubiquinone. Complex IV activity was determined by measuring the oxidation of cytochrome *c* at 550 nm. Protein content was determined by the Bradford method as described above [47], and ETC activity values were expressed as nmol substrate consumed/min/mg protein. ATP synthase activity was measured as oligomycin-sensitive ATPase activity using an assay coupled with pyruvate kinase, which converts ADP to ATP and produces pyruvate from phosphoenolpyruvate as previously described [46, 52-54]. Final values were expressed as nmol of NADH oxidized/min/mg protein.

2.9 Western blot analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was run on 4-12% gradient gels as previously described [42, 55] with equal amounts of protein loaded. Relative amounts of mitofilin, cytochrome *c* oxidase (COX IV), mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), OPA1,

CHCHD3, dynamin-related protein 1 (Drp1) and GAPDH were assessed using the following primary antibodies; anti-mitofilin rabbit antibody (product no. ab48139, Abcam, Cambridge, MA), anti-COX IV rabbit antibody-mitochondrial loading control (product no. ab16056, Abcam, Cambridge, MA), anti-Mfn1 rabbit antibody (product no. ab104585, Abcam, Cambridge, MA), anti-Mfn2 rabbit antibody (product no. ab50838, Abcam, Cambridge, MA), anti-OPA1 rabbit antibody (product no. ab42364, Abcam, Cambridge, MA), anti-CHCHD3 rabbit antibody (product no. ab98975, Abcam, Cambridge, MA), anti-Drp1 rabbit antibody (product no. sc32898, Santa Cruz Biotechnology, Dallas, TX), and anti-GAPDH rabbit antibody (product no. ab8245, Abcam, Cambridge, MA). The secondary antibody used was goat anti-rabbit IgG horseradish peroxidase conjugate (product no. 10004301, Cayman Chemical Company, Ann Arbor, MI). Detection of signal was performed using a Pierce ECL Western blotting substrate detection system according to the manufacturer's directions (Thermo Fisher Scientific Inc., Rockford, IL). Quantification of chemiluminescent signals were assessed using a G:Box Bioimaging System (Syngene, Frederick, MD), and data were expressed as arbitrary optical density units. Densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD). Protein loading was further confirmed using Ponceau staining in addition to COX IV and GAPDH, where appropriate.

2.10 ETC Complex Protein Expression

To assess ETC complex abundances, blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as previously described [39, 45] with modifications according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) using equal amounts of protein. Briefly,

isolated mitochondria were solubilized with 1% digitonin on ice. After addition of Coomassie G-250, samples were run on 4–16% NativePAGE gels. Following BN-PAGE, gels were placed in a fixed solution containing 40% methanol and 10% acetic acid followed by microwaving for 45 seconds at 1,100 watts. Gels were then washed for 15 minutes at room temperature after which the solution was decanted. Destaining was accomplished by addition of 50 ml of an 8% acetic acid solution and microwaved a second time for 45 seconds at 1,100 watts. The gel was then shaken at room temperature until the desired background was obtained. To control for destaining time and enable comparison between gels, each band of interest was expressed per the molecular weight marker 480 kDa band. The gel was then scanned and densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD).

2.11 Mitochondrial DNA (mtDNA) content

Examination of mtDNA content was performed on whole heart lysate from control and mitofilin transgenic mice using a NovaQUANTTM mouse mtDNA to nuclear DNA ratio kit that compares the levels of nuclear DNA to mtDNA. As per the manufacturers protocol (Millipore, Billerica, MA), an RTPCR platform was utilized to compare several nuclear and mitochondrial genes and the mtDNA copy number was quantified per diploid nuclear genome. An Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies, Grand Island, NY) was used for the analyses.

2.12 Mitochondrial size, internal complexity and membrane potential

Mitochondrial size, internal complexity and membrane potential ($\Delta\Psi_m$) were measured as previously described by our laboratory with modifications [34, 42, 46]. Briefly, flow cytometric analyses were performed using a FACS Calibur equipped with a 15-MW 488-nm argon laser and 633-nm red diode laser (Becton Dickinson, San Jose, CA). Each individual parameter (gating, size, and complexity) was performed using specific light sources (laser and photomultiplier tube) and specific detectors. MitoTracker deep red 633 (Invitrogen, Carlsbad, CA), which moves into intact mitochondria due to membrane potential, was used to selectively stain intact mitochondria (emission wavelength: 633 nm, fluorescent 633 red diode laser) and exclude debris, enabling accurate gating of intact mitochondria. Freshly isolated mitochondria were incubated with the MitoTracker dye and subsequently assessed for size and internal granularity. Forward scatter (FSC) and side scatter (SSC) detectors were used to examine approximate size (FSC; absolute particle size) and approximate internal complexity (SSC; refracted and reflected light which is proportional to granularity of the object) in isolated mitochondria. Internal complexity or granularity of isolated mitochondria was expressed with respect to size (arbitrary unit) of the mitochondria as FSC/SSC ratio. The ratiometric dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes, Carlsbad, CA), was used to assess mitochondrial membrane potential. Changes in membrane potential were recorded as the ratio between the color shifts from green to orange. All flow cytometric measurements were performed in conjunction with the West Virginia University Flow Cytometry Core Facility.

2.13 Lipid peroxidation

Lipid peroxidation by-products malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE), stable end products formed from the oxidation of polyunsaturated fatty acids and esters, were assessed as previously described [34, 46]. Absorbance was measured on a Molecular Devices Flex Station 3 spectrophotometer (Molecular Devices, Sunnyvale, CA), and protein content was assessed as described above [47] with final values expressed per milligram of protein.

2.14 Electron microscopy

A section of left ventricle was cut and fixed for electron microscopy images. Briefly, specimens were washed in 0.2M phosphate buffer, post-fixed by incubation for 2 hours with 2% osmium tetroxide (Electron Microscopy Science, Hatfield, PA), dehydrated in a graded series of ethanol solutions (from 50% to 100%) and propylene oxide, and embedded in Epon resin (SPI Supplies, Westchester, PA). Embedded samples were allowed to polymerize for 48 hours at 62°C. Ultrathin sections (50nm) were cut from the resulting blocks with a Leica Ultracut UCT ultramicrotome (Leica Biosystems, Buffalo Grove, IL) and then captured on 200 mesh copper electron microscopy grids. The sections were observed at 120kV with a Zeiss Libra 120 electron microscope (Carl Zeiss NTS, LLC, Peabody, MA, USA) connected to a Gatan Orius SC 1000 CCD digital camera driven by Digital Micrograph software (Gatan, Pleasanton, CA) for image acquisition and analysis. All electron microscopy imaging was performed in conjunction with the West Virginia University Tissue Processing and Analysis Core Facility.

2.15 Statistics

Means \pm SE were calculated for all data sets. Data were analyzed using a one way ANOVA (GraphPad software, La Jolla, CA). A Tukey comparison of all groups was used as the post-hoc test to determine the significant differences among means. A Student's *t*-test was utilized when evaluating mitofilin content in mouse and human mitochondria. $P < 0.05$ was considered significant.

3. Results

3.1 Mitofilin transgenic mouse characterization

Two mitofilin transgenic mouse lines were created, mitofilin Tg Line 1 and mitofilin Tg Line 2. DNA from mitofilin Tg Line 1 displayed average CT values of 26.5, while mitofilin Tg Line 2 displayed average CT values of approximately 21, indicating that Line 2 possessed greater mitofilin cDNA content. In contrast, DNA from transgenic negative mice of all lines displayed CT values of approximately 34-36 which is similar to the CT value of water. As a result, we chose to perform all experimentation using mitofilin Tg Line 2 and its associated littermate controls.

To verify increased protein expression in our transgenic mice, mitofilin protein content was determined in both whole heart homogenate and isolated mitochondrial subpopulations. Transgenic SSM and IFM subpopulations displayed significantly higher levels of mitofilin protein content when compared to littermate controls which was not significantly impacted by diabetic insult (Figure 3.2 A-D). In addition, mitofilin protein levels were significantly higher in transgenic whole heart homogenate as compared to littermate controls (Figure 3.3). Evaluation of mitofilin protein content in control and diabetic mitochondria revealed significant decrements in type 1 diabetic IFM with no significant decrease in diabetic SSM (Figure 3.4 A-D; $P < 0.05$). Because down-regulation of mitofilin has been associated with loss of mtDNA content, we determined whether mtDNA levels were influenced by overexpression in our transgenic mouse model [3]. Examination of mtDNA content in mitofilin transgenic and littermate controls revealed no significant differences (Littermate Control 5319.83 ± 2265.05 copies vs. Mitofilin Transgenic 4866.88 ± 1995.13 copies). To determine whether type 1 diabetic patient heart

mitochondria displayed similar mitofilin profiles as a result of the pathology, we examined mitofilin protein contents in non-diabetic and type 1 diabetic human patient atrial tissue. Our data reveal significant decreases in IFM mitofilin protein contents in diabetic patient atrial tissue as compared to non-diabetic patients with no significant differences in SSM between diabetic and non-diabetic patients (Figure 3.5 A-D).

3.2 Body weight, heart weight and cardiac contractile function

Significant decreases in heart and body weights were observed in diabetic and mitofilin diabetic animals when compared with their respective controls (Table 3.1; $P < 0.05$). However, there was no significant differences observed in heart weight to body weight ratios. Cardiac contractile dysfunction was observed in diabetic hearts when compared with control hearts. Ejection fraction, fractional shortening and cardiac output were significantly decreased in the type 1 diabetic heart relative to control (Table 3.1; $P < 0.05$). Overexpression of mitofilin in the presence of diabetes mellitus restored ejection fraction and fractional shortening (Table 3.1; $P < 0.05$). Further, no significant differences were observed in stroke volume, or volumes at systole and diastole between any groups. Finally, no changes in diameter at systole and diastole were observed.

3.3 Mitochondrial functional assessment

Assessment of ETC complexes I, III, IV and V activities revealed significant decrements in diabetic IFM as compared with control IFM (Table 3.2; $P < 0.05$), with no significant

differences between control and diabetic SSM (Table 3.2). Overexpression of mitofilin preserved ETC complexes I, III, IV and V activities in mitofilin diabetic IFM as compared to diabetic IFM (Table 3.2; $P<0.05$). A significant increase in ATP synthase activity was also observed in the mitofilin SSM subpopulation as compared to control SSM (Table 3.2; $P<0.05$). Using complex I substrates glutamate/malate, state 3 and state 4 respiration rates were significantly decreased in diabetic IFM compared to control IFM (Table 3.2; $P<0.05$), with no significant differences observed between control and diabetic SSM (Table 3.2). Overexpression of mitofilin in the diabetic heart restored state 3 respiration rates in the IFM (Table 3.2; $P<0.05$) with no significant effects observed in the SSM (Table 3.2).

3.4 ETC Complex Protein Expression

To gain insight into whether the observed decreases in ETC complex activities were the result of down-regulation of individual complex contents, we employed a BN-PAGE approach (Figure 3.6 A) which enabled evaluation of the expression of each ETC complex as a whole. Our data indicated no significant differences in complexes I, III, IV, and V contents between the four treatment groups in SSM (Figure 3.7 A-D) and IFM (Figure 3.8 A-D).

3.5 Mitochondrial membrane potential ($\Delta\Psi_m$)

No significant differences in SSM $\Delta\Psi_m$ were observed among the four groups, (Figure 3.9A), however, there was a significant reduction of $\Delta\Psi_m$ in the diabetic IFM subpopulation compared with control which was restored with mitofilin overexpression (Figure 3.9B; $P<0.05$).

3.6 Oxidative stress

Lipid peroxidation by-products 4-HAE and MDA were significantly increased in diabetic IFM compared to control IFM (Figure 3.10B; $P < 0.05$), with no significant differences observed in diabetic SSM as compared to control SSM (Figure 3.10A). Mitofilin overexpression significantly decreased the accumulation of lipid peroxidation by-products in diabetic IFM, restoring them back to that of control IFM levels (Figure 3.10B; $P < 0.05$), suggesting an ability of mitofilin to attenuate ROS-induced damage to lipids.

3.7 Electron microscopy

Qualitative analysis of mitochondrial morphology was performed by electron microscopy. Visualization of IFM structure was assessed in control, diabetic, mitofilin control and mitofilin diabetic heart. Morphologically altered IFM with damaged cristae structures were observed in the diabetic heart when compared to the control heart (Figures 3.11A-B). Overexpression of mitofilin restored mitochondrial morphology and cristae structure in the mitofilin diabetic IFM (Figure 3.12B), suggesting a potential role for mitofilin in the maintenance of cristae morphology following type 1 diabetic insult.

3.8 Mitochondrial internal complexity

Because of the improved mitochondrial morphology and cristae structure observed within the electron micrographs of mitofilin diabetic IFM, we determined whether internal complexity of the mitochondria were affected by mitofilin expression using flow cytometry. FSC and SSC

were used to estimate size and internal complexity of mitochondria respectively (Figures 3.13A-B). The ratio of SSC to FSC was calculated in an effort to determine changes in internal complexity with respect to size. Using this approach, diabetic IFM showed significantly decreased internal complexity when compared with control (Figure 3.13D; $P < 0.05$) with no changes observed in the SSM (Figure 3.13C). Overexpression of mitofilin significantly restored mitochondrial internal complexity in mitofilin diabetic IFM (Figure 3.13D; $P < 0.05$). Restoration of mitochondrial morphology and cristae structure with mitofilin overexpression could potentially account for the improvement in mitochondrial internal complexity in mitofilin diabetic IFM.

3.9 Mitochondrial dynamics

Because type 1 diabetes mellitus and mitofilin have been associated with mitochondrial dynamics and cristae morphological changes, we examined several ancillary proteins involved in the regulation of these processes. Western blot analyses indicated no significant differences in any of the proteins that we examined (Mfn1, Mfn2, OPA1, CHCHD3, and Drp1) (Figures 3.14 and 3.15) which are associated with mitochondrial dynamics, suggesting that mitofilin overexpression did not alter the levels of these proteins.

4. Discussion/Conclusion

A number of studies corroborate the notion that mitochondrial dysfunction plays a critical role in the pathogenesis of the diabetic heart. Previous studies from our laboratory suggest that during type 1 diabetic insult mitochondria are spatially impacted with those situated in between the myofibrils (IFM) affected to a greater extent [33, 34, 41, 42]. In particular, the IMM are disturbed as evidenced by decreased ETC complex activities, ATP synthase activity, protein import, cardiolipin content and cardiolipin synthase activity correlating with the manifestation of mitochondrial dysfunction [33, 34, 40-42]. Further, mitochondrial morphology including changes in size and internal complexity of IFM are also observed. Proteomic alterations during type 1 diabetic insult, which are disproportionately realized in the IMM of the IFM subpopulation, have been previously reported [33]. These alterations include a decrease in mitofilin, a mitochondrial structural protein. Among mitofilin's primary functions is the maintenance of cristae junctions, branching of cristae and preservation of IMM morphology [3, 17]. Preservation of cristae structure is essential for proper mitochondrial function and health due to the presence of ETC complexes vital for ATP production which are contained in the IMM [5, 6]. The goal of this study was to determine whether preservation of mitofilin provides cardioprotective benefits to the type 1 diabetic heart and whether the beneficial effects are associated with improved mitochondrial cristae structure. Overexpression of mitofilin in the SSM and IFM of transgenic mice revealed significantly higher expression of the protein in IFM as compared to SSM. These findings are in agreement with Ferreira *et al.* who showed a 37 fold increase of mitofilin in cardiac tissue IFM when compared with SSM [56].

Proteomic analyses from our laboratory reveal a significant decrease in mitofilin content in IFM during type 1 diabetic insult [33]. Further, morphological alterations are observed

primarily in the IFM subpopulation [34, 57]. Thus, it is plausible that the decreased mitofilin content observed in the IFM, resulting from type 1 diabetes mellitus may account, in part, for the mitochondrial morphological changes observed which could have downstream effects on mitochondrial functionality ultimately leading to cardiac contractile dysfunction. Electron micrographs from diabetic hearts revealed damaged mitochondrial cristae in mitochondria situated between the myofibrils when compared with the control hearts. Moreover, a significant decrease in mitochondrial internal complexity and granularity was observed in diabetic IFM. Our findings are in agreement with John *et al.* who investigated the impact of mitofilin down-regulation in HeLa cells. Their findings revealed a disorganized IMM as well as increased ROS production and apoptosis [16]. Moreover, their study revealed that mitofilin-deficient mitochondria fail to produce normal tubular cristae and cristae junctions. These author's observations share similarities to the structural abnormalities observed in diabetic IFM in our current study. In a similar study, Rabl *et al.* investigated the down-regulation of Fcj1, a putative orthologue of mammalian mitofilin, in yeast cells. The results of these studies revealed a lack of cristae junctions which was correlated with abnormal mitochondrial structure. Electron microscopy analyses from hearts overexpressing mitofilin revealed preservation of mitochondrial morphology and cristae structure in type 1 diabetic IFM. Moreover, restoration of mitochondrial internal complexity and granularity was observed in mitofilin diabetic IFM. Our findings are in agreement with a study in which overexpression of Fcj1 in yeast cells led to increased cristae junction formation and cristae branching, suggesting normal mitochondrial structure [3]. Taken together, data from the current study showing abnormal mitochondrial structure as a result of decreased mitofilin content in diabetic IFM, which was restored with mitofilin overexpression, are in agreement with others [3, 16, 17]. Finally, overexpression of mitofilin led to preservation

of ejection fraction and fractional shortening, suggesting improved cardiac contractile function which may be the result of improvement in mitochondrial structure and ultimately, function.

The inner mitochondrial cristae membrane is the principal site for oxidative phosphorylation and ATP production [5]. Mitochondrial cristae structure increases the inner membrane surface area enabling a greater capacity for oxidative phosphorylation and ATP production. In the current study diabetic IFM with disrupted mitochondrial cristae structure and decreased internal complexity displayed significant decrements in ETC complexes I, III, IV as well as ATP synthase function which are in agreement with previous observations from our laboratory [34, 39]. Overexpression of mitofilin restored ETC complexes and ATP synthase activities in the mitofilin diabetic IFM which were associated with restoration of cristae morphology as well as mitochondrial internal complexity. Though not assessed in the current study, these findings may have been the result of an increased surface area leading to an enhanced capacity for oxidative phosphorylation and ATP generation. Nevertheless, it should be noted that mitofilin overexpression did not lead to changes in the absolute contents of ETC complexes. These findings suggest that the benefits imparted by mitofilin overexpression are not the result of increased ETC complexes, rather preservation of IMM integrity which could enhance stabilization of the ETC complexes. Additional studies designed to determine changes in cristae surface area and their impact on ETC stabilization in the face of enhanced mitofilin presence would lend insight into the mechanisms contributing to the observed oxidative phosphorylation changes.

Several studies have shown enhanced ROS production from cardiac mitochondria of different type 1 diabetic animal models [34, 58]. The oxidative milieu resulting from enhanced ROS production promotes damage to mitochondrial membranes and proteins. We have

previously observed increased oxidative damage as indexed through nitrotyrosine residues and lipid peroxidation in type 1 diabetic IFM [34]. In the current study, we observed similar increases in lipid peroxidation of diabetic IFM. Interestingly, overexpression of mitofilin attenuated lipid peroxidation in diabetic IFM which could be due to improved mitochondrial structure, and preservation of ETC function. Preservation of mitochondrial structure could potentially attenuate electron leakage resulting in decreased ROS production and downstream oxidative damage.

In contrast to our study, mitofilin was reported to be increased in pathological cardiac hypertrophy and was shown to promote cardiac hypertrophy in response to hypertrophic stimuli. In the current study we observed a decrease in heart weight with no change in heart weight/body weight ratio as a result of a parallel decrease in body weight following diabetes mellitus induction. One potential limitation of our study was the lack of assessment of heart weight/tibia length which may have been decreased provided tibia length remained constant which has been reported in previous low-dose STZ studies [59]. Moreover, mitofilin overexpression was shown to increase ROS generation and lower oxidative phosphorylation activity in animals subjected to a cardiac hypertrophy protocol [60]. The differences in the observed results between the two studies could be due to a multitude of reasons. Zhang *et al.* utilized whole tissues for assessing oxidative phosphorylation and frozen tissues for their ROS measure in comparison with isolated mitochondria used in our studies. Moreover, two different models of cardiomyopathy were being studied; diabetic cardiomyopathy and hypertrophy that depict a differential expression of mitofilin levels suggesting its distinct role in these pathologies. Furthermore, decrements in mitofilin content have also been observed in other human diseases such as Down's syndrome [24, 25], Parkinson's disease [26, 27], Epilepsy [28, 29] and Neurodegeneration [30, 31]. Hence,

these varied changes of mitofilin observed in different human pathologies as well as different cardiac pathologies suggest unique responses and effects of mitofilin under different pathological stimuli.

In conclusion, we report for the first time, that overexpression of mitofilin in a type 1 diabetic mouse model preserves mitochondrial structure and function, resulting in cardiac contractile protection. Mitochondrial functional preservation is associated with the restoration of ETC complex activities, ATP synthase activity, state 3 respiration rates, and attenuation of lipid peroxidation which are the result of improved mitochondrial morphology, structure and internal complexity.

Conflict of Interest

There are no conflicts of interest to disclose.

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Figure 3.1

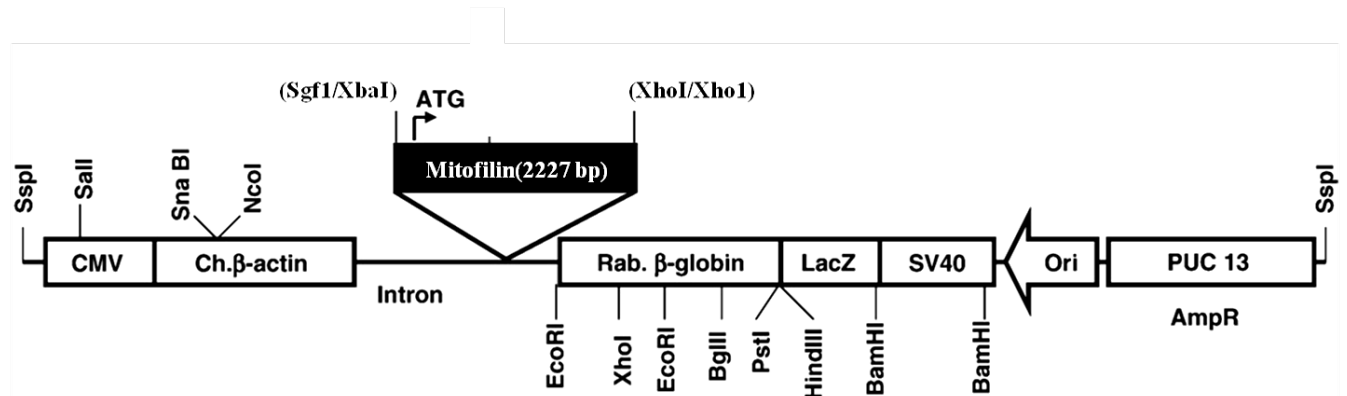


Figure 3.1. Schematic of mitofilin transgenic construct. The generation of mitofilin transgenic mice was accomplished by insertion of human mitofilin cDNA into the XbaI and XhoI cloning sites of pCAGGS as SgfI and XhoI fragment and released by SspI and BamHI digestion.

Figure 3.2

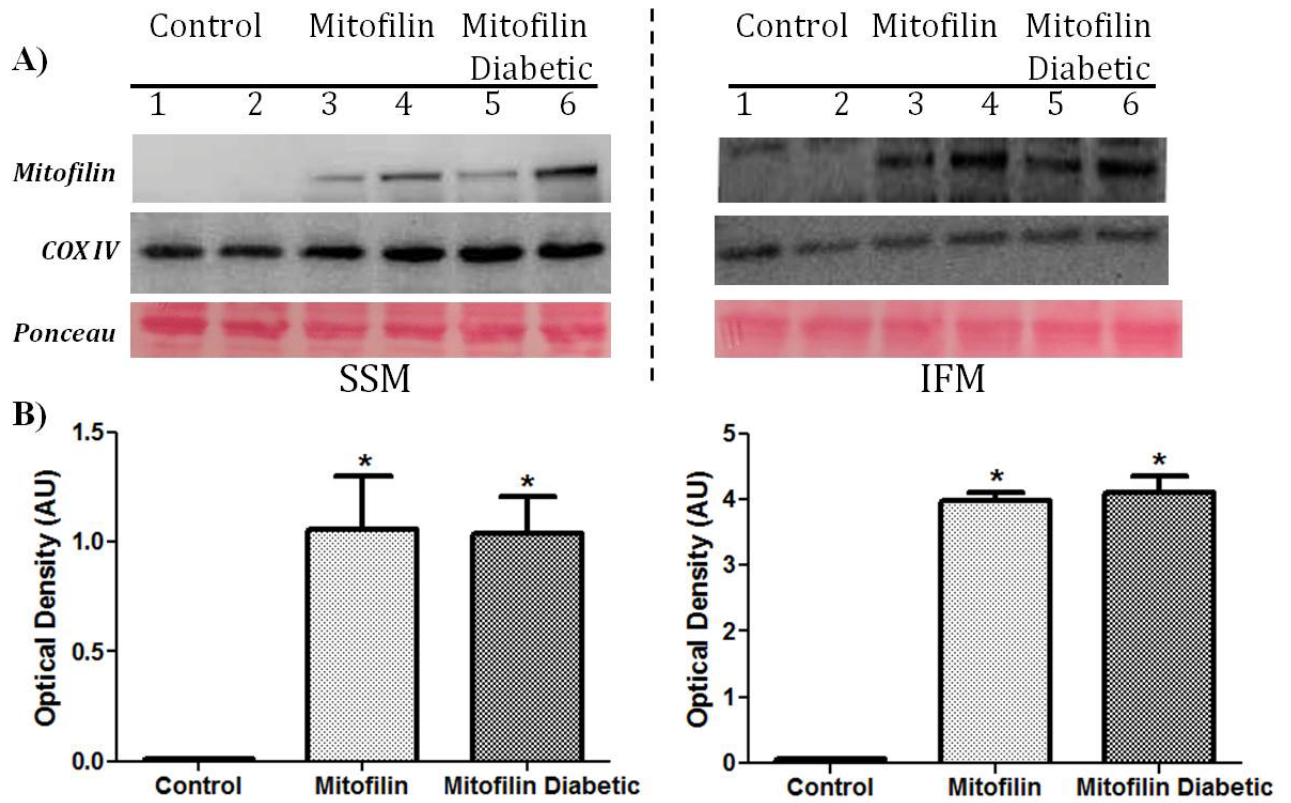


Figure 3.2. Mitofilin protein expression in control and transgenic mitochondrial subpopulations. Western blot analysis of mitofilin protein expression in isolated (A) SSM and IFM mitochondria. Lanes 1-2 are controls, lanes 3-4 are mitofilin transgenic, and lanes 5-6 are mitofilin transgenic diabetic samples for both SSM and IFM mitochondria. (B) Bar graph representation for SSM and IFM mitochondria protein levels. COX IV staining was used as loading controls for the blots. Values are expressed as means \pm SEM. *P <0.05 vs. control; n=4 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.3

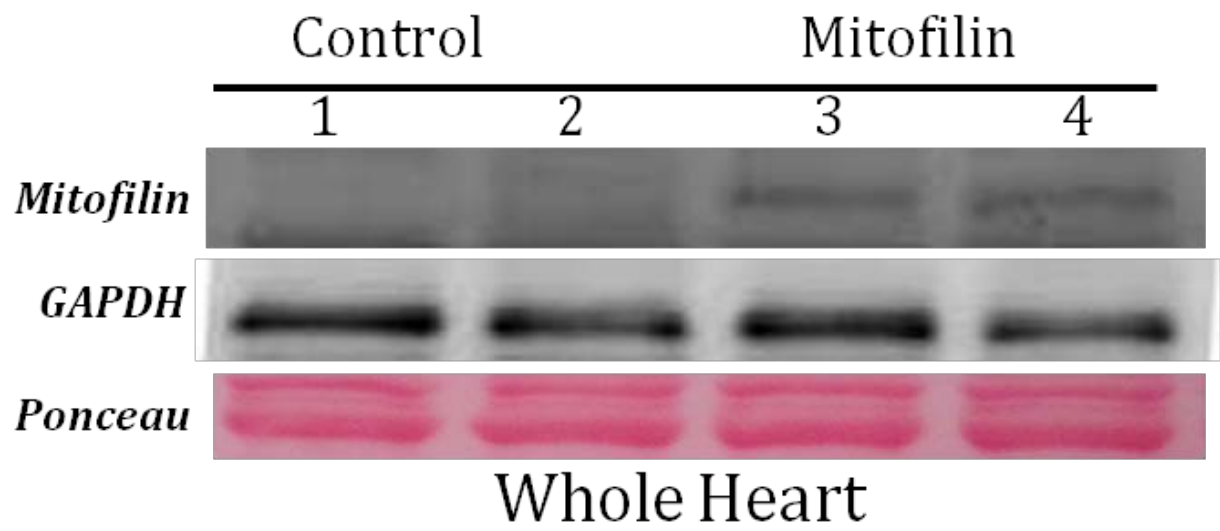


Figure 3.3. Mitofilin protein expression in control and transgenic whole heart. Western blot analysis of mitofilin protein expression in total heart mitochondria from control (lanes 1-2) and mitofilin transgenic (lanes 3-4) mouse. GAPDH and ponceau staining were used as loading controls for the blots. Values are expressed as means \pm SEM. *P <0.05 vs. control; n=4 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.4

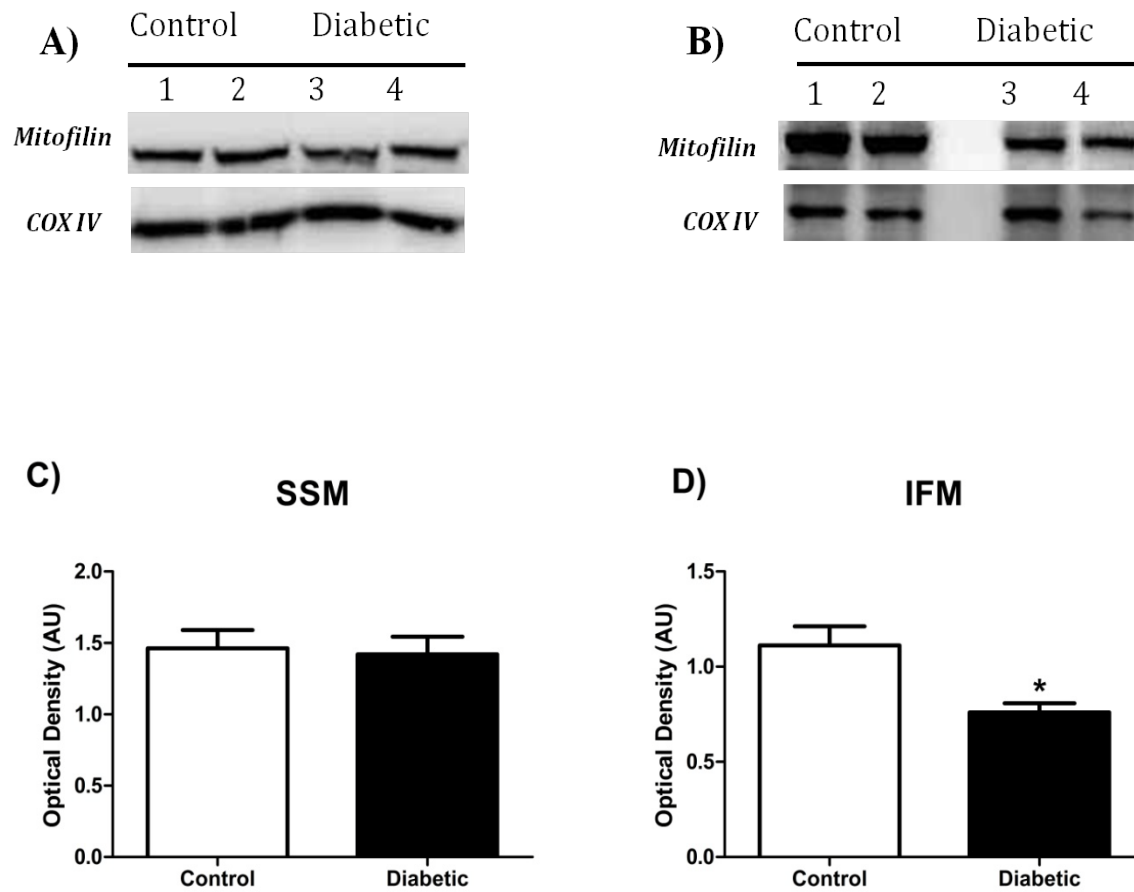


Figure 3.4. Mitofilin protein expression analysis in control and diabetic mitochondria subpopulations from mouse. (A) Control SSM (lanes 1 and 2) and diabetic SSM (lanes 3 and 4) (B) control IFM (lanes 1 and 2) and diabetic IFM (lanes 3 and 4) were analyzed for mitofilin protein expression. Bar graph representation of mitofilin protein levels over COX IV in SSM (C) and IFM (D) subpopulations. Values are presented as means \pm SE; *P <0.05 for control vs. diabetic. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=5 for each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.5

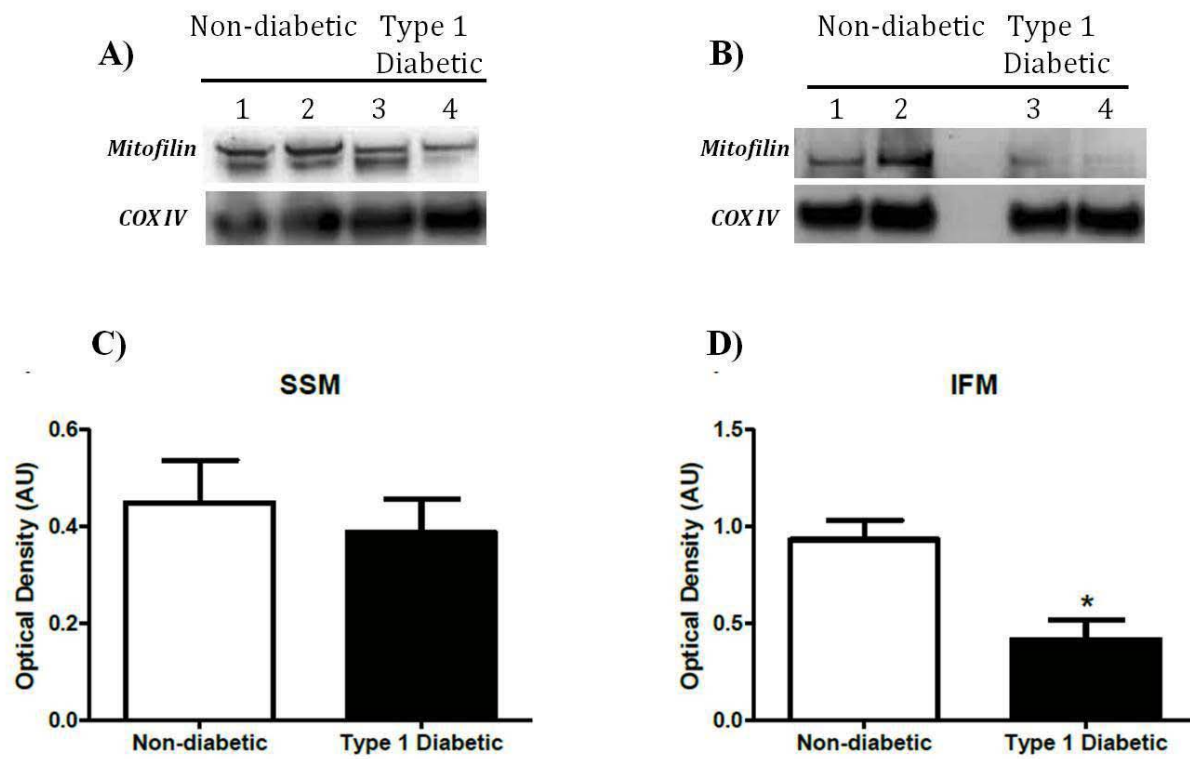


Figure 3.5. Mitofilin protein expression analysis in control and diabetic mitochondria subpopulations from human samples. Analysis of mitofilin protein content in non-diabetic and type 1 diabetic human patient samples in SSM (A) and IFM (B) subpopulations. Bar graph representation of mitofilin protein levels over COX IV in SSM (C) and IFM (D) isolated from human patient samples. Values are presented as means \pm SE; *P <0.05 for control vs. diabetic. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=5 for each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.6

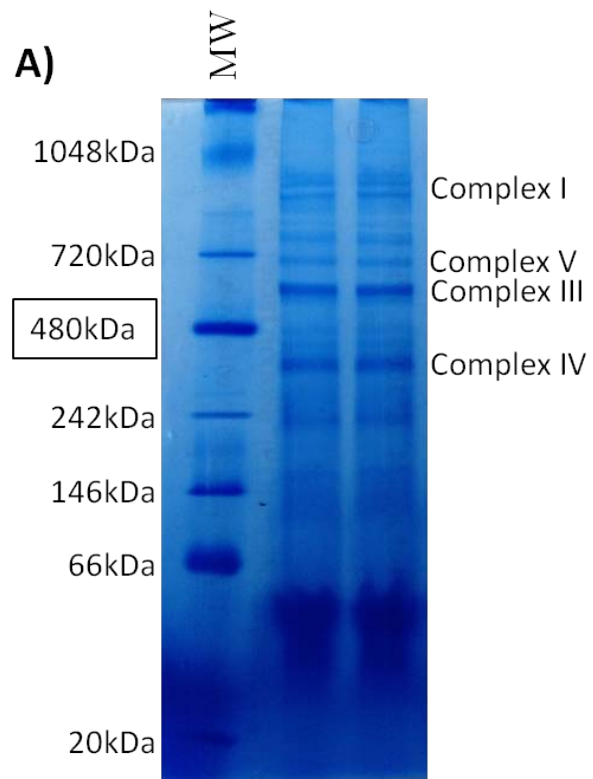


Figure 3.6. Blue Native page analyses. (A) Molecular weight markers in kDa along with ETC complexes size are included for two control samples. MW: molecular weight marker.

Figure 3.7

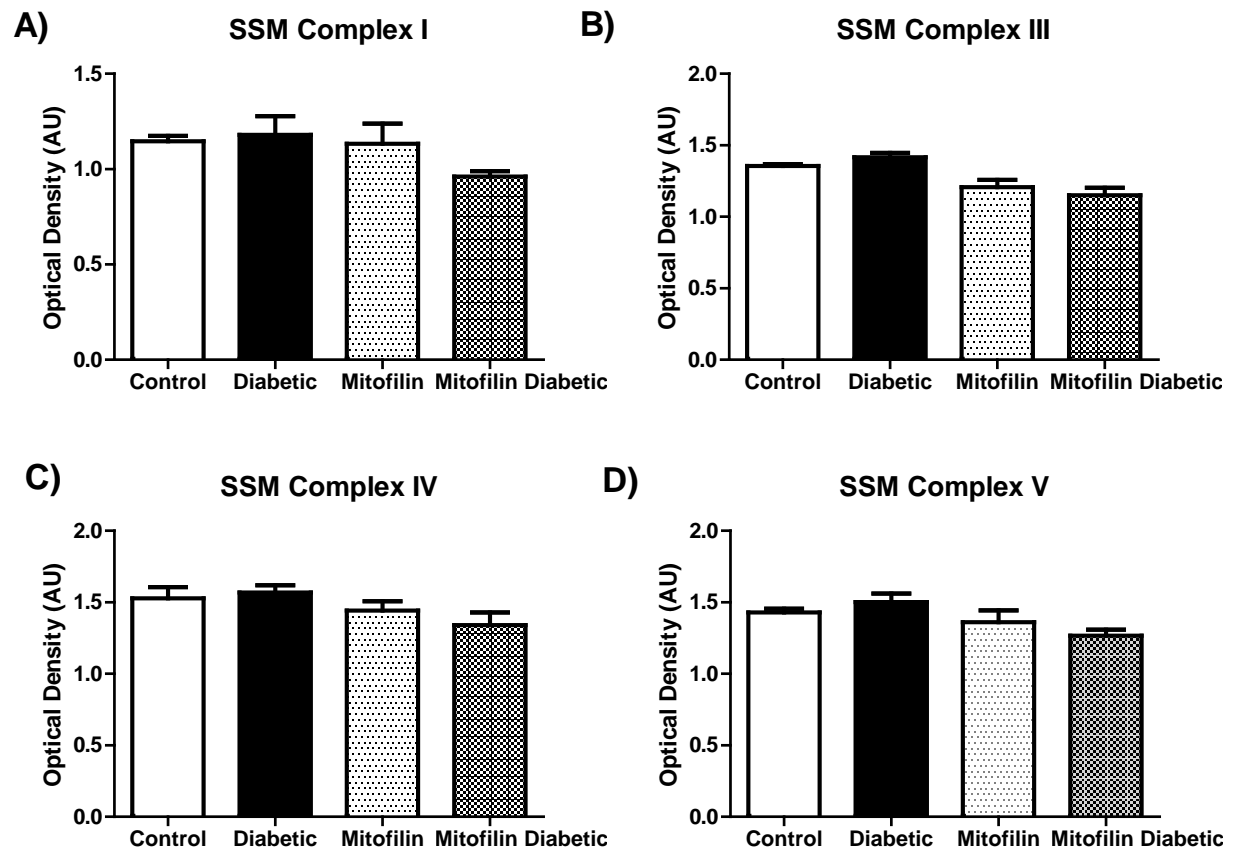


Figure 3.7. BN-PAGE analysis of ETC Complexes in SSM subpopulations. ETC complexes expression was examined in control, diabetic, mitofilin control and mitofilin diabetic mitochondrial SSM subpopulations using BN-PAGE. Bar graph representation of ETC complexes I, III, IV and V assessed for SSM (A-D). Values are expressed as means \pm SEM. n=6 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.8

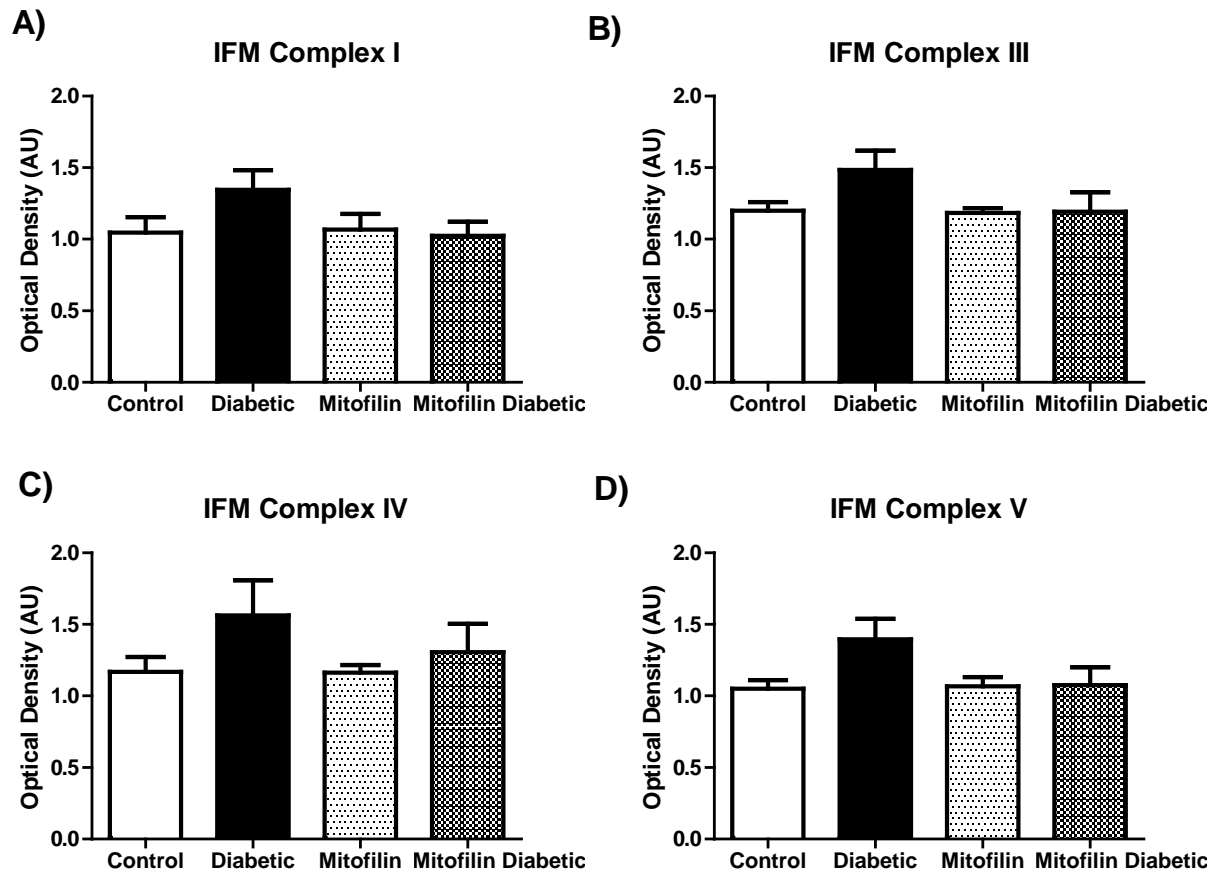


Figure 3.8. BN-PAGE analysis of ETC Complexes in IFM subpopulations. ETC complexes expression was examined in control, diabetic, mitofilin control and mitofilin diabetic mitochondrial IFM subpopulations using BN-PAGE. Bar graph representation of ETC complexes I, III, IV and V assessed for IFM (A-D). Values are expressed as means \pm SEM. n=6 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.9

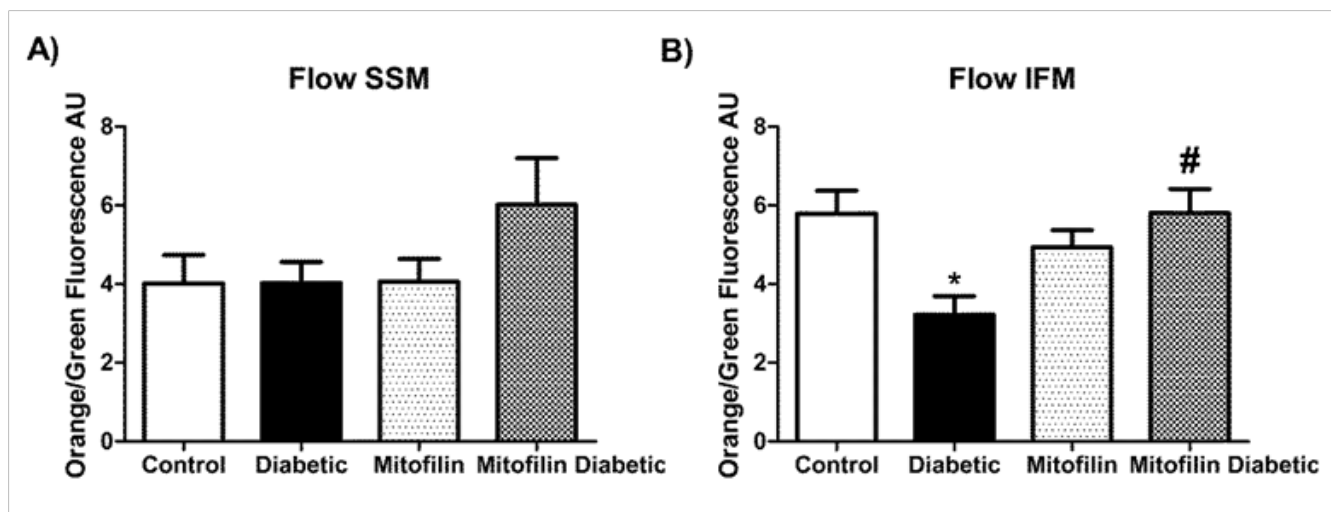


Figure 3.9. Mitochondrial membrane potential. Isolated mitochondria from control, diabetic, mitofilin control and mitofilin diabetic hearts were incubated with JC-1, and 100,000 gated events were analyzed per sample in (A) SSM and (B) IFM subpopulations. Values are expressed as means \pm SEM. * $P < 0.05$ for control vs. diabetic and # $P < 0.05$ for diabetic vs. mitofilin diabetic; n=8 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.10

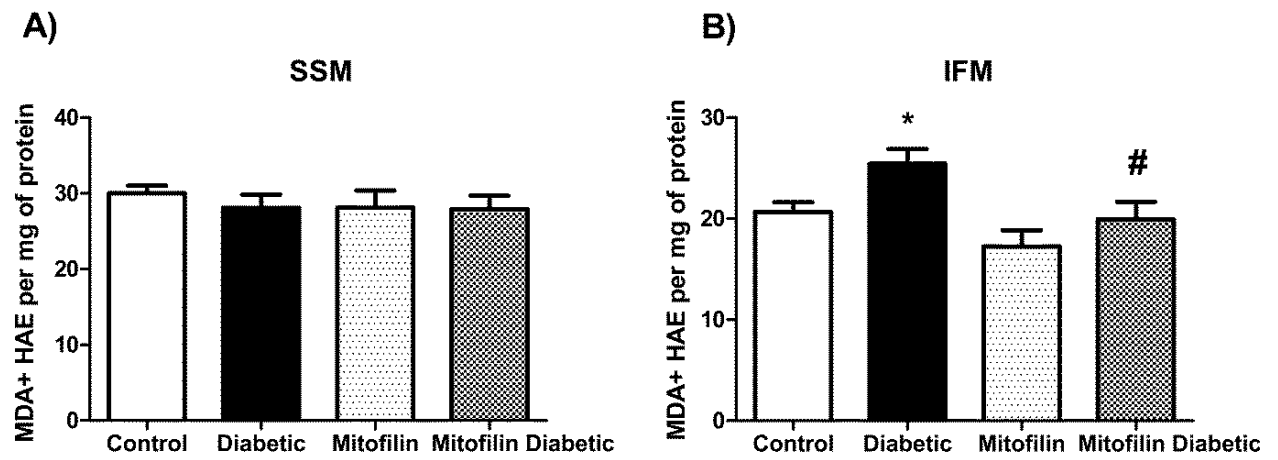
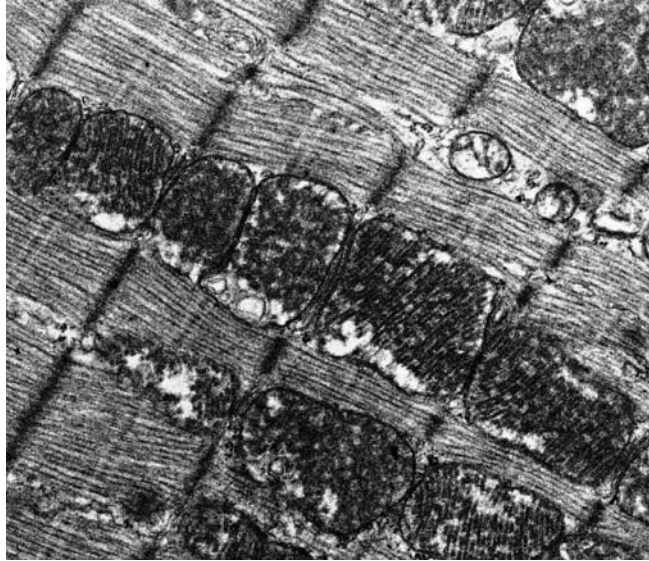


Figure 3.10. Lipid peroxidation by-products. Oxidative damage to lipids was assessed in control, diabetic, mitofilin control and mitofilin diabetic (A) SSM and (B) IFM subpopulations by measuring lipid peroxidation by-products malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAE) using a colorimetric assay. Results were compared against a standard curve of known 4-HAE and MDA concentrations. Values are expressed as means \pm SEM. * $P < 0.05$ for control vs. diabetic and [#] $P < 0.05$ for diabetic vs. mitofilin diabetic; n=4 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.11

A)



B)

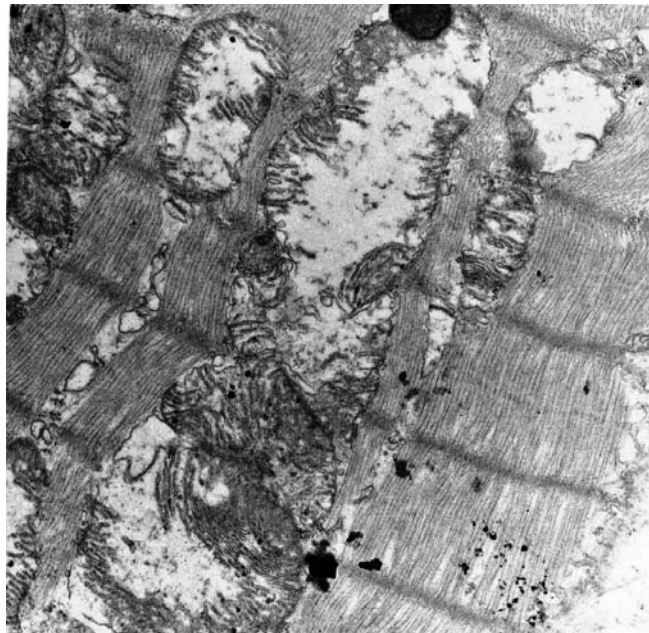
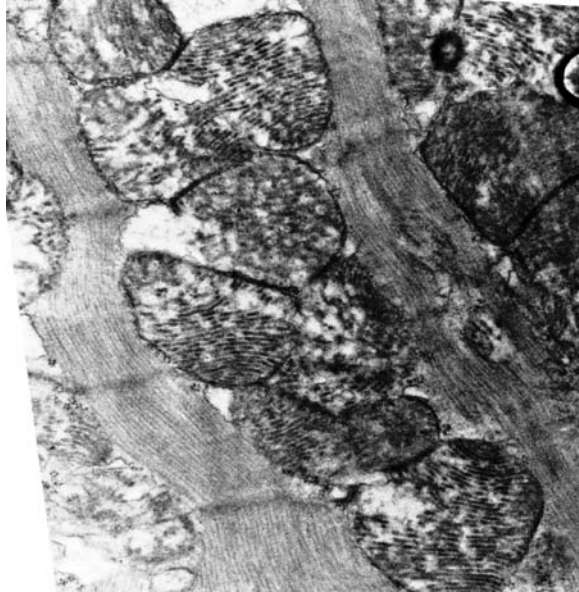


Figure 3.11. Mitochondrial structure. Electron micrographs(x 16.0K) from left ventricle of (A) Control, and (B) Diabetic mice hearts showing mitochondrial morphology and cristae structures in the IFM subpopulations of type 1 diabetic heart. IFM: interfibrillar mitochondria
➤ : myofibrils.

Figure 3.12

A)



B)

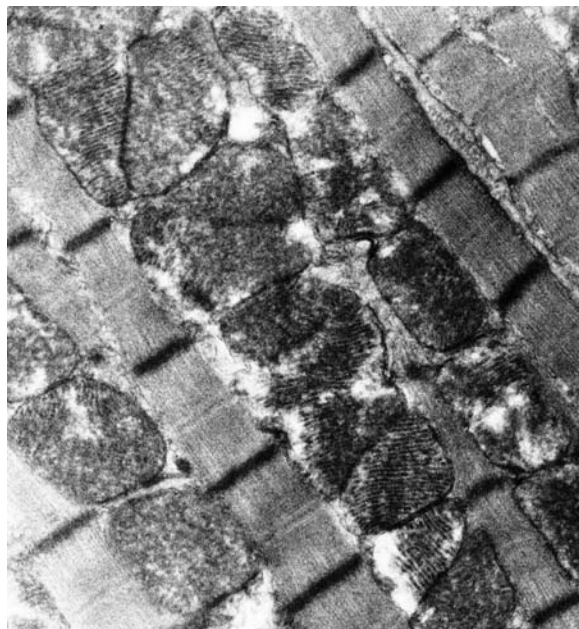


Figure 3.12. Mitochondrial structure. Electron micrographs(x 16.0K) from left ventricle of (A) Mitofilin Control, and (B) Mitofilin Diabetic mice hearts showing mitochondrial morphology and cristae structures in the IFM subpopulations of type 1 diabetic heart. IFM: interfibrillar mitochondria ➤ : myofibrils.

Figure 3.13

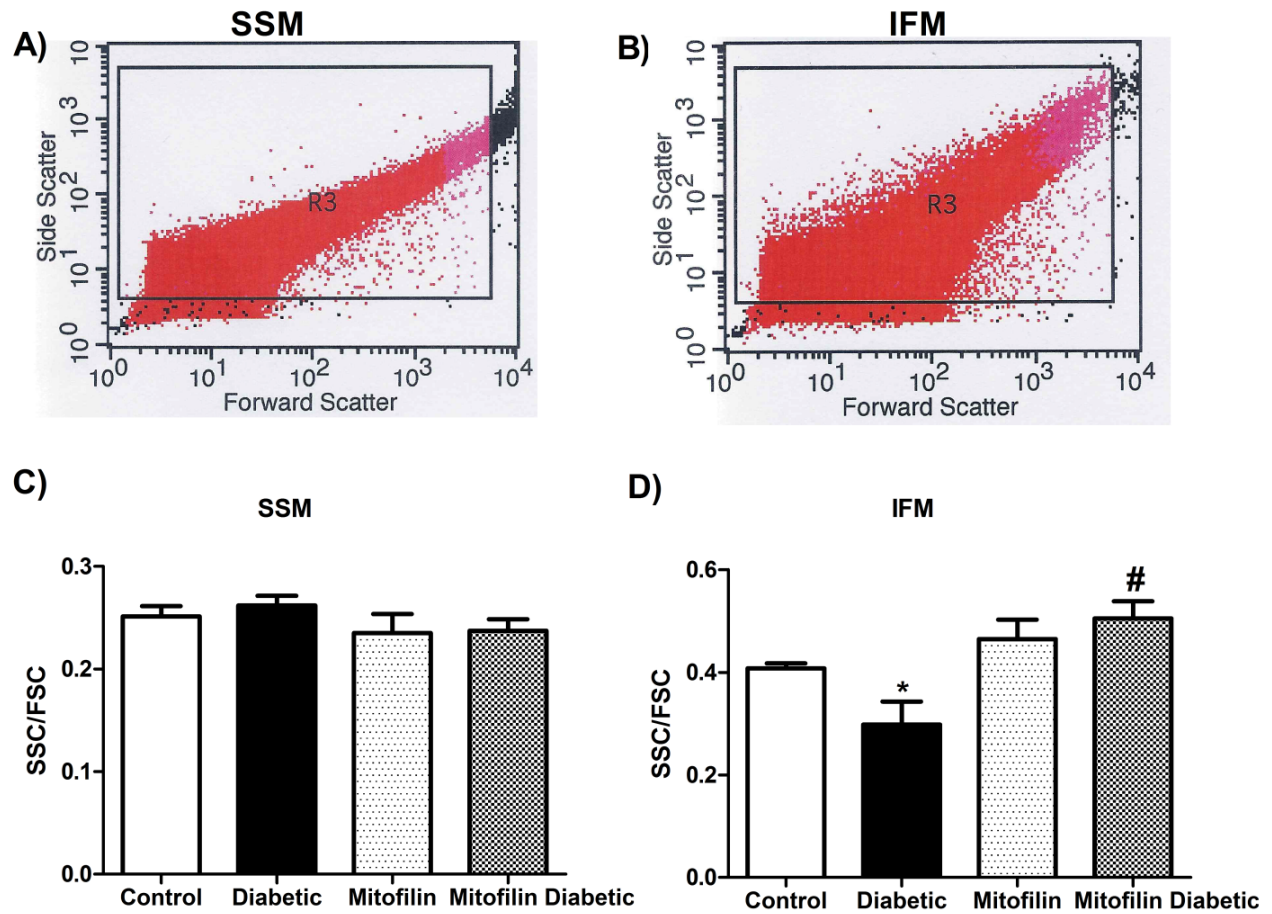


Figure 3.13. Mitochondrial Internal Complexity. Mitochondrial internal complexity was assessed in control, diabetic, mitofilin control and mitofilin diabetic hearts. Forward scatter and side scatter were used to analyze isolated mitochondria as seen by representative histograms of (A) SSM and (B) IFM. The ratio of side scatter to forward scatter was used to calculate internal complexity of the mitochondria in (C) SSM and (D) IFM mitochondria. Values are expressed as means \pm SEM. * $P < 0.05$ for control vs. diabetic and # $P < 0.05$ for diabetic vs. mitofilin diabetic; n=8 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 3.14

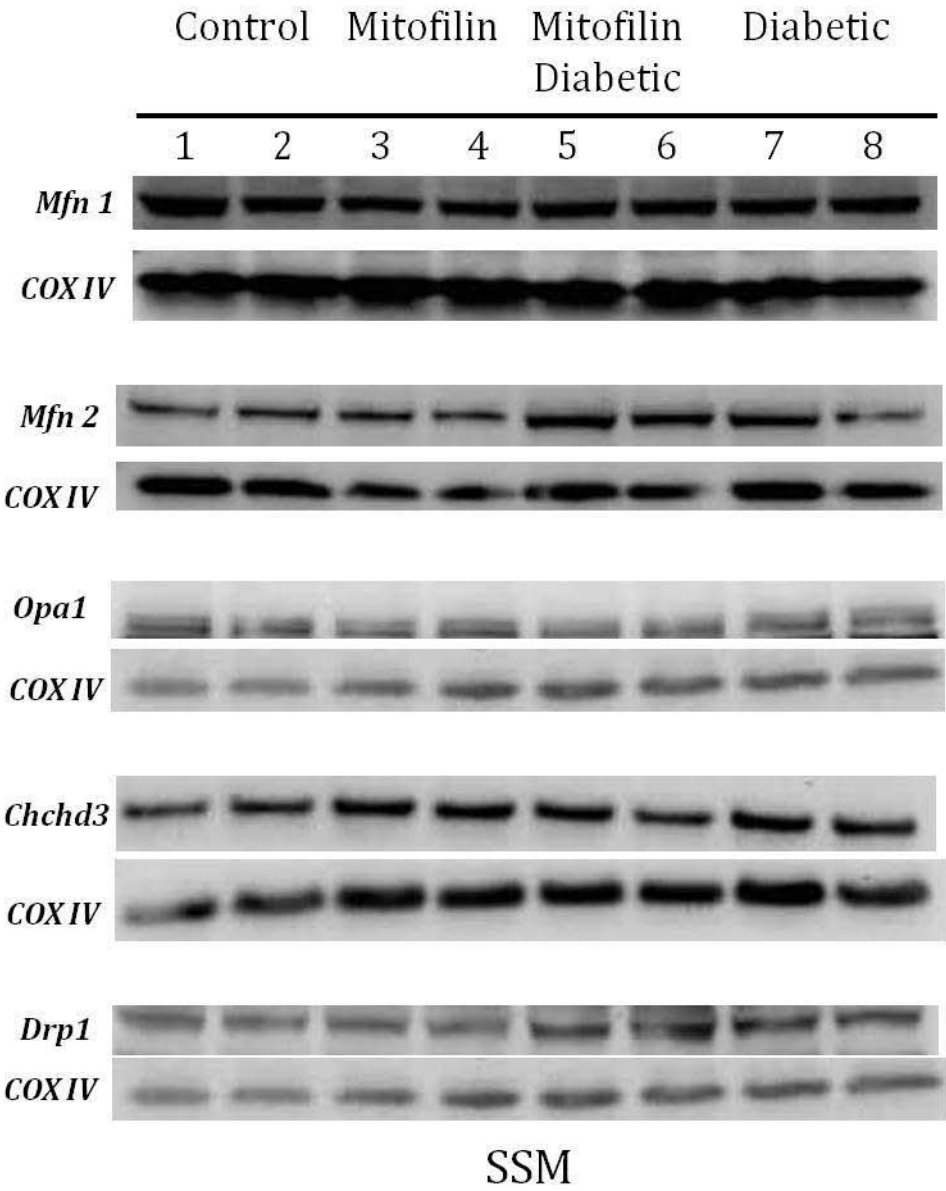


Figure 3.14. Western Blot analyses of mitochondrial dynamics. Protein expression of mitochondrial fission, fusion, Chchd3 and Opa1 proteins were analyzed in control, mitofilin, mitofilin diabetic, and diabetic cardiac SSM mitochondria. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=4 for each group. SSM: subsarcolemmal mitochondria.

Figure 3.15

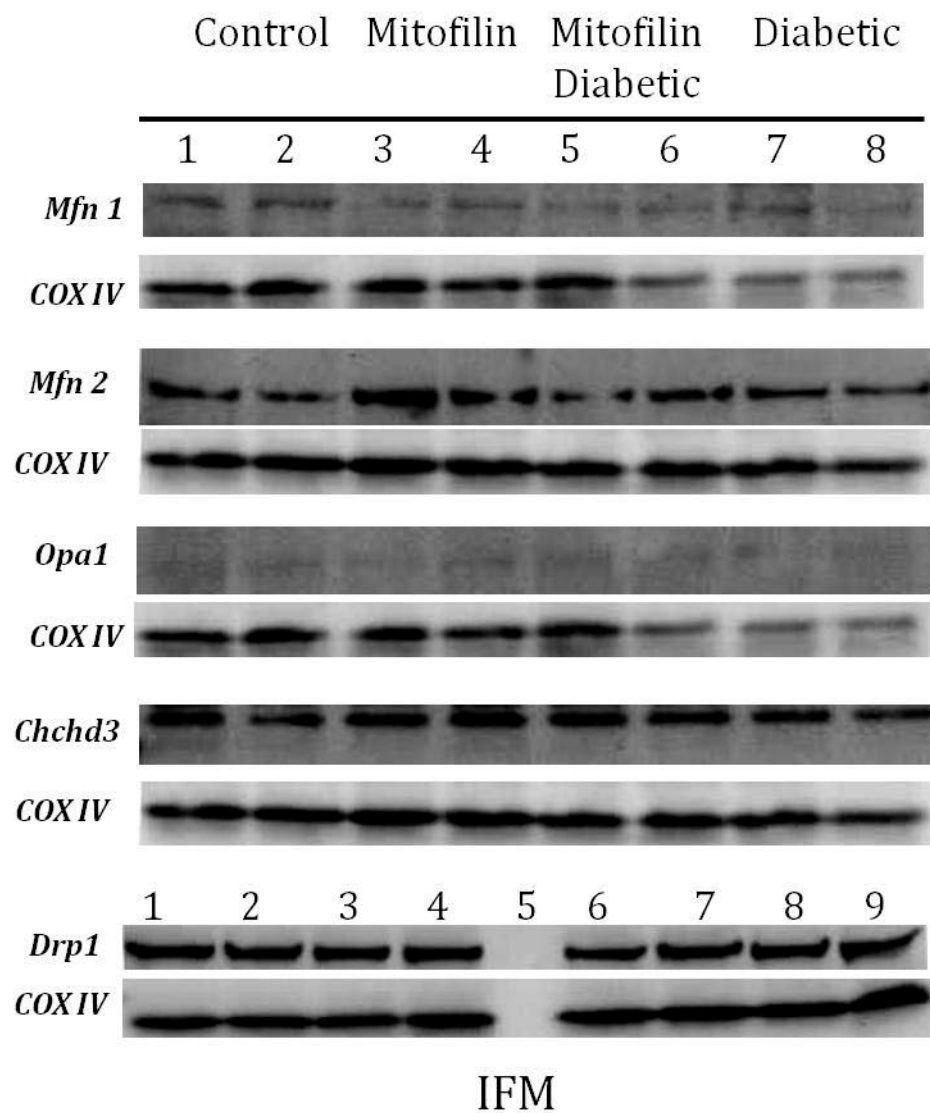


Figure 3.15. Western Blot analyses of mitochondrial dynamics. Protein expression of mitochondrial fission, fusion, Chchd3 and Opa1 proteins were analyzed in control, mitofilin, mitofilin diabetic, and diabetic cardiac IFM mitochondria. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=4 for each group. IFM: interfibrillar mitochondria.

Table 3.1

Contractile Parameter	Control	Diabetic	Mitofilin Control	Mitofilin Diabetic
BW, g	28.0 ± 0.8	25.5 ± 0.5 [*]	27.1 ± 0.7	24.6 ± 0.4 [*]
HW, mg	102.7 ± 4.4	94.3 ± 2.5 [*]	103.0 ± 2.1	91.3 ± 1.9 [*]
HW/BW, mg/g	3.7 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Ejection fraction, %	70.6 ± 1.4	65.2 ± 0.8 [*]	70.6 ± 1.8	74.7 ± 1.5 [#]
Fractional shortening, %	39.0 ± 1.1	34.8 ± 0.6 [*]	39.4 ± 1.4	42.4 ± 1.4 [#]
Cardiac Output, ml/min	18.1 ± 1.2	14.2 ± 1.4 [*]	17.5 ± 1.1	16.0 ± 2.7
Diameter; s, mm	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.2	1.8 ± 0.1
Diameter; d, mm	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.1 ± 0.1
Volume; s, µl	13.8 ± 0.6	14.4 ± 1.5	12.8 ± 2.6	10.1 ± 1.0
Volume; d µl	41.4 ± 4.0	44.7 ± 3.5	47.4 ± 4.3	37.9 ± 2.4
Stroke volume, µl	32.3 ± 1.9	28.1 ± 2.3	34.6 ± 2.1	28.3 ± 1.7
Heart rate, bpm	517.0 ± 15.7	487.0 ± 13.6	506.0 ± 14.5	486.0 ± 21.6

Table 3.1. Cardiac contractile function. Cardiac contractile measurements were assessed using the Vevo 2100 imaging system. Values are means \pm SEM. * $P < 0.05$ diabetics vs. their respective control, [#] $P < 0.05$ diabetic vs. mitofilin diabetic; n=20 for BW, HW and n=6 for other parameters. BW: body weight, HW: heart weight, s: systole, d: diastole, bpm: beats per minute.

Table 3.2

Group	Complex I	Complex III	Complex IV	Complex V	State 3	State 4
SSM Control	32.2 ± 4	98.7 ± 17	12.5 ± 2.4	12.1 ± 0.9	16.1 ± 1.1	2.6 ± 0.5
SSM Diabetic	29.2 ± 2.7	88 ± 7.6	10.7 ± 1.5	12.9 ± 0.9	18.1 ± 0.7	1.9 ± 0.3
SSM Mitofilin	30.4 ± 3.7	86 ± 11.7	10.4 ± 1.9	$16.8 \pm 1.4^*$	17.9 ± 2.1	3.6 ± 0.6
SSM Mitofilin Diabetic	29.9 ± 1.9	88.4 ± 13.7	12 ± 2.6	14.7 ± 1.2	22.2 ± 2.6	2 ± 0.3
IFM Control	46.8 ± 1.9	197 ± 8.8	39 ± 7	24.5 ± 1.3	21.8 ± 0.9	$4.2 \pm 0.6^{\dagger}$
IFM Diabetic	$31.3 \pm 2.8^*$	$151 \pm 6.6^*$	$19.5 \pm 2^*$	$20.6 \pm 0.6^*$	$14.7 \pm 0.6^*$	2 ± 0.2
IFM Mitofilin	44.1 ± 6.1	173 ± 13.6	31.9 ± 2.7	23.9 ± 0.7	19.2 ± 1.4	2.4 ± 0.3
IFM Mitofilin Diabetic	$45.9 \pm 1.7^{\#}$	$206 \pm 11^{\#}$	$37.7 \pm 4^{\#}$	$26.6 \pm 1.1^{\#}$	$22.3 \pm 3.5^{\#}$	2.2 ± 0.1

Table 3.2. Mitochondrial functional assessment. Mitochondrial electron transport chain (ETC) complex activities and respiration rates were examined in control, diabetic, mitofilin control and mitofilin diabetic mitochondrial subpopulations. ETC complexes I, III, IV and V activities were assessed spectrophotometrically by measuring the oxidation of NADH (complex I), reduction of cytochrome c (complex III), oxidation of cytochrome c (complex IV) and an assay coupled with pyruvate kinase (complex V). Enzymatic activities for complexes I, III and IV are expressed as activity per minute per milligram of protein and complex V is expressed as nmol of NADH per minute per milligram of protein. State 3 and state 4 respiration rates were determined in the presence of the substrates glutamate-malate, and state 3 respiration was examined upon addition of ADP. Values are expressed as means \pm SEM. * $P < 0.05$ vs. control, [#] $P < 0.05$ vs. diabetic and [†] $P < 0.05$ against all groups; n=8 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Chapter 4:

Translational Regulation of the Mitochondrial Genome Following Redistribution of Mitochondrial MicroRNA (MitomiR) in the Diabetic Heart

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Running Title: mitomiR regulation of the mitochondrial genome

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ABSTRACT

Rationale: Cardiomyocytes are rich in mitochondria which are situated in spatially-distinct subcellular regions including those under the plasma membrane (subsarcolemmal mitochondria; SSM), and those between the myofibrils (interfibrillar mitochondria; IFM). Previously, we have observed subpopulation-specific differences in mitochondrial proteomes following diabetic insult.

Objective: Determine whether mitochondrial genome-encoded proteins are regulated by microRNAs present in the mitochondrion and whether mitochondrial subcellular spatial location or diabetes mellitus influences the dynamics.

Methods and Results: Using microarray technology coupled with cross-linking immunoprecipitation and next generation sequencing, we identified a functional pool of mitochondrial microRNAs, termed mitomiRs that are redistributed in spatially-distinct mitochondrial subpopulations in an inverse manner following diabetic insult. Redistributed mitomiRs displayed distinct interactions with the mitochondrial genome requiring specific stoichiometric associations with RISC constituents argonaute-2 (Ago2) and fragile X mental retardation-related protein 1 (FMR1) for translational regulation. In the presence of Ago2 and FMR1, redistribution of mitomiR-378 to the IFM following diabetic insult led to down regulation of mitochondrially-encoded F0 component (ATP6). Next generation sequencing analyses identified specific transcriptome and mitomiR sequences associated with ATP6 regulation. Overexpression of mitomiR-378 in HL-1 cells resulted in its accumulation in the mitochondrion

and down-regulation of functional ATP6 protein, while antagomir blockade restored functional ATP6 protein and contractile function.

Conclusions: We propose that mitomiR translational regulation of mitochondrially-encoded proteins oscillates in spatially-distinct mitochondrial subpopulations during diabetes mellitus. The results reveal the requirement of RISC constituents in the mitochondrion for functional mitomiR translational regulation and provide a connecting link between diabetic insult and ATP synthase function.

Keywords: Mitochondria Diabetes Mellitus microRNA

ABBREVIATIONS

Ago2 = argonaute-2

ARE = AU rich elements

CLIP = cross-linked immunoprecipitation

FPKM = fragments per kilobase of exon per million

FXR1 = fragile X mental retardation–related protein 1

IFM = interfibrillar mitochondria

miRNA = microRNA

mitomiR = mitochondrial microRNA

RISC = RNA-induced silencing complex

SSM = subsarcolemmal mitochondria

INTRODUCTION

Emerging technologies are likely to aid in the discovery of the underlying gene regulation linking the mitochondrion to disease initiation and progression. Cardiac tissue is rich in mitochondria with spatially-distinct subpopulations which include subsarcolemmal mitochondria (SSM), located beneath the cell membrane, and interfibrillar mitochondria (IFM), situated between the myofibrils ^{1, 2}. Type 1 diabetes mellitus influences cardiac contractility of which mitochondrial dysfunction is central ²⁻⁵. The complete mitochondrial genomic sequence has been elucidated ⁶ with each mitochondrion possessing between two and ten copies ⁷. The mitochondrial genome is comprised of 37 genes, 13 of which code for proteins that form parts of the oxidative phosphorylation machinery, while the remaining genes code for 22 transfer RNAs and two ribosomal RNAs ⁸. Absence of introns renders the mitochondrial DNA compact while lack of histones influences mutation rates and DNA damage repair mechanisms ⁷. As such, the development and progression of many human diseases involves regulation of the mitochondrial genome ^{9, 10}.

MicroRNAs (miRNAs) have been shown to play an integral role in regulating gene expression through translational repression or degradation of target messenger RNAs (mRNA). miRNAs are non-coding RNAs, which influence post-transcriptional gene regulation by binding to the 3'-untranslated region (3'-UTR) of target mRNAs. Regulation of gene expression at the post-transcriptional level occurs through binding of the miRNA to complementary sites on the target mRNA. Association with the RNA-induced silencing complex (RISC) takes place in the cytoplasm, thereby regulating translation of the target mRNA ¹¹. Target mRNAs can contain AU

rich elements (ARE) which, in conjunction with a given miRNA, will enhance recruitment of the RISC components, including argonaute-2 (Ago2) and fragile X mental retardation-related protein 1 (FMR1), thereby influencing translation¹². Mature miRNAs have been observed in the nucleus and specialized processing bodies¹³, yet reports identifying mature miRNAs in the mitochondrion are limited^{14, 15}. A recent review supporting the existence of miRNAs in the mitochondrion have utilized the terminology mitomiR in reference to mitochondrial miRNAs¹⁶. Pharmacological inhibition of cardiac-specific miRNA enhances metabolic energy homeostasis suggestive of a link between miRNA regulation and the mitochondrion¹⁷. Further, mice lacking specific miRNAs, including miR-378, exhibit enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity¹⁸. Though increasing evidence indicates that mitochondrial subpopulations are differentially influenced by physiological stressors^{3, 19-22}, no study has reported specific enrichment patterns of mitomiRs in spatially-distinct subpopulations or whether distribution patterns are influenced by pathological states, including diabetes mellitus. The results of our studies suggest a dynamic system requiring the presence of RISC constituents in the mitochondrion, which function to regulate the mitochondrial genome during diabetic insult in a spatially-distinct manner.

METHODS

Ethics Statement

The experiments in this study conformed to the *NIH Guide for the Care and Use of Laboratory Animals (8th Edition)* and were approved by the West Virginia University Animal Care and Use Committee.

Experimental Animals and Diabetes Induction

Male FVB mice (The Jackson Laboratory, Bar Harbor, ME) were housed in the West Virginia University Health Sciences Center animal facility and given food and water *ad libitum*. Type 1 diabetes mellitus was induced in 5 week old mice using multiple low-dose streptozotocin (STZ; Sigma, St. Louis, MO) injections as previously described^{2, 3}. Mice that served as vehicle controls were given the same volume per body weight of sodium citrate buffer. One week post-injections, hyperglycemia was confirmed by measuring fasting blood glucose levels using a commercially available kit (Bayer, Mishawaka, IN). Blood glucose levels greater than 250 mg/dL were considered diabetic.

Preparation of Individual Mitochondrial Subpopulations

At 5 weeks post-hyperglycemia onset, mice were sacrificed and their hearts excised. SSM and IFM subpopulations were isolated as previously described¹ with minor modifications^{2, 3, 23-25}. Following isolation, SSM and IFM were further purified by percoll gradient (23%, 15%, 10% and 3% percoll solution) and centrifuged in a Beckman OptimaTM MAX-XP Ultracentrifuge (Beckman Coulter, Fullerton, CA) at 32,000 x g for 8 minutes. Mitochondrial subpopulation

pellets were resuspended in a sucrose based SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) or mitochondrial extraction buffer (Biovision, Mountain View, CA) depending upon the assay to be performed. Mitochondrial protein concentrations were determined using the Bradford method and bovine serum albumin as a standard ²⁶.

RNA Isolation and Microarray Experimentation

Total RNA was extracted from isolated mitochondrial subpopulations using a Vantage Total RNA Purification Kit (Origene, Rockville, MD). Sample labeling and hybridization was performed using an Agilent miRNA Complete Labeling and Hybridization Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. Briefly, 80 ng of total RNA was labeled and subsequently hybridized overnight (20 hours at 55°C) to an Agilent Mouse microRNA Microarray 8x60K v.16 using Agilent's recommended hybridization chamber and oven. The microarrays were washed once with Agilent Gene Expression Wash Buffer 1 (5 minutes at room temperature) followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (5 minutes at 37°C). Fluorescence signals were detected using Agilent's Microarray Scanner System (Agilent Technologies, Santa Clara, CA). Differential miRNA expression FES derived output data files were analyzed using Gene Spring GX software (Agilent Technologies, Santa Clara, CA). Candidate miRNAs were identified and analyzed statistically.

Computational Analyses

To scan the mitochondrial genome for potential miRNA target sites, we used three independent algorithms, RNAhybrid, miRWalk and MicroCosm. The algorithmic core of

RNAhybrid does not utilize any RNA folding or pairwise sequence alignment code, but rather, implements an algorithm that was specifically designed for RNA hybridization (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html>). The miRWalk algorithm is based on a computational approach starting with a heptamer seed miRNA seed, and identifies complementation on the complete mitochondrial genome (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>). The MicroCosm target algorithm uses a weighted scoring system and rewards complementation at the 5' end of the miRNA (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/info.html>). A probability distribution of random matches for a subsequence in the given sequence is calculated. All prediction algorithms were run under default parameters.

Network Analyses

Network analyses were performed on altered miRNAs from the microarray analyses and on the predicated targets identified from the Computational Analyses (see above). Mitochondrially-encoded genes were mapped to corresponding gene objects in the Ingenuity Knowledge Base (<http://ingenuity.com/>). These mitochondrial genes, called focus genes, were overlaid into a global miRNA network developed from the information contained in the Ingenuity Pathways Knowledge Base. Networks of focus genes were then algorithmically generated based on their connectivity. A network pathway is a graphical representation of the molecular relationships between individual miRNAs as well as miRNAs and mitochondrially-encoded mRNAs.

qRT-PCR of Mature miRNAs

Briefly, 50 ng of total RNA was reverse transcribed into cDNA using a First-strand cDNA Synthesis Kit for miRNA (Origene, Rockville, MD) as per the manufacturer's protocol. Equal amounts of cDNA from control and diabetic mouse mitochondrial subpopulations were subjected to qRT-PCR. For HL-1 and miR-378 overexpressing HL-1 cell lines, isolated mitochondria were subjected to qRT-PCR. Custom primers were designed for miR-378 (Origene, Rockville, MD) and utilized for the quantification of cDNA replicates using QSTAR SYBR master mix (Origene, Rockville, MD). qRT-PCR assays were performed in duplicate using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). All miRNA expression levels were normalized to U6 RNA contents.

Western Blot Analyses

SDS-PAGE was run on 4–12% gradient gels, as previously described ^{2, 27}, with equal amounts of protein loaded for each study treatment. Relative amounts of subpopulation-specific mitochondrially-encoded proteins were quantified using antibodies specific for each protein (antibody details are indicated in the Supplemental Material section). Relative amounts of subpopulation-specific mitochondrial localized RISC complex proteins, Ago2 and FXR1, as well as biotinylated proteins were quantified using the following primary antibodies; anti-Ago2 rabbit antibody (product #2897, Cell Signaling, Danvers, MA), anti-FXR1 rabbit antibody (product #4264S, Cell Signaling, Danvers, MA), anti-GFP mouse monoclonal (product #TA150041, Origene, Rockville, MD) and anti-biotin rabbit antibody (product #5571, Cell Signaling, Danvers, MA). Mitochondrial loading control was assessed using an anti-COX IV rabbit antibody (product #ab16056, Abcam, Cambridge, MA). The secondary antibody used in the analyses was a goat anti-rabbit IgG HRP conjugate (product #10004301, Cayman Chemical,

Ann Arbor, MI). Detection and quantitation of chemiluminescent signals were performed using a G:BOX (Syngene, Frederick, MD), and the data expressed as arbitrary optical density units (ODU). Secondary confirmation of protein loading and equal protein transfer was performed using Ponceau staining.

ATP Synthase Activity

ATP synthase activity was determined by following the decrease in NADH absorption at 340 nm as previously described²⁵ and final values expressed as micromoles consumed per minute per milligram of protein²⁶.

UV Cross-Linking Immunoprecipitation (CLIP)

Mouse heart tissue was minced in a 2 ml volume of ice-cold PBS, transferred to a petri dish with a suspension depth of approximately 1 mm. Samples were irradiated 5 times with 400 mJ/cm² (~15 cm distance from the UV source) on ice using a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA), and mixed between each irradiation²⁸. After irradiation, samples were pelleted by centrifugation (2500 rpm for 5 minutes at 4°C) and the supernatant removed. SSM and IFM were isolated as described above and resuspended in IP buffer (20mM Tris pH 8.0; 137mM NaCl; 10% Glycerol; 1% Nonidet P-40 (NP-40); 2mM EDTA; 1/100 protease inhibitor cocktail). Protein concentrations were determined as above. Two hundred µg of resuspended protein was added to IP buffer up to 1 ml and the RNA digested by addition of 10 µl of RNase I (Life Technologies, Grand Island, NY) (1:500 dilution). The complex was incubated (3 minutes at 37°C) with constant shaking (1,100 rpm) then transferred to ice. Fifty µl of Dynabeads Protein A (Life Technologies, Grand Island, NY) were washed 3 times with IP

buffer and then resuspended in a 100 μ l of solution of IP buffer and 5 μ l of a specific antibody (anti-Ago2 or anti-FXR1). The antibody was allowed to bind to the beads by rotating the tubes (1 hour at room temperature). The beads were then washed three times with IP buffer, and cross-linked mitochondrial subpopulation lysates added followed by tube rotation (2 hours at 4°C). Beads were then washed three times with IP buffer. A portion of the sample was utilized to check antibody specificity using high RNase I (1:50 dilution), while the other portion was used for RNA 3'-end biotinylation labeling (Thermo Scientific, Pittsburgh, PA) using T4 RNA ligase to attach a single biotinylated nucleotide to the 3' terminus of an RNA strand. Samples were incubated overnight at 16°C and then washed with IP buffer. Thirty μ l of loading buffer without denaturing agent was added to both the biotinylated and high RNase I treated beads and heated for 10 minutes at 70 °C with shaking (1000 rpm). Samples were submitted to SDS-PAGE as described above. The biotinylated (low RNase I) sample bands were cut with a clean scalpel and the nitrocellulose membrane placed into a microcentrifuge tube. One hundred μ l of proteinase K solution was added to each tube, incubated for 20 minutes at 37 °C with shaking (1000 rpm), and then the RNA extracted using a Vantage Total RNA Purification Kit (Origene, Rockville, MD) as above. qRT-PCR was performed using custom primers designed for targets of mouse miR-378 as described above as well as mitochondrially-encoded genome primer duplex targets (Origene, Rockville, MD). Data were normalized to CLIP total RNA input and RISC proteins (Ago2 and FXR1).

Next Generation Sequencing

Four groups of pooled CLIP-RNA samples (control and diabetic; SSM and IFM; n=5 per group) were used for high-throughput sequencing. mRNA and miRNA library preparation was

performed using true RNA-seq (Illumina, San Diego, CA) and small RNA-seq kits (Illumina, San Diego, CA), respectively. Briefly, as per the manufacturer's instructions, initial RT-PCR, and barcoded products were run on a 2% agarose gel and 200- to 400-bp products purified using QiaEx II beads (Qiagen, Valencia, CA). Gel-purified material was amplified for 12 cycles with fusion Illumina adaptor primers and the DNA was subjected to next generation sequencing. The sequencing of CLIP tags was performed with barcoded libraries using the Illumina Sequencing (Mi-Seq) Adaptor primers. Base calling, library sorting by barcode, and mapping to the transcriptome were performed, subsequently.

Bioinformatic Analysis of CLIP Tags

CLIP tags were aligned to the mm10 genome with mitochondrial genome and analyzed with Avadis NGS which is an integrated platform that provides tools for extensive workflows for alignment, analysis, management and visualization of next-generation sequencing data (<http://www.avadis-ngs.com/>). To crosscheck the Avadis NGS results, we employed several other platforms including Galaxy (<http://galaxy.psu.edu>), the UCSC genome browser (<http://genome.ucsc.edu/>), and miRBASE (<http://microrna.sanger.ac.uk>). RNA abundance from the paired-end RNA-Seq data was expressed as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). To assess RISCome enrichment and depletion scores between the diabetic and control SSM and IFM, diabetic RISCome FPKM values were normalized to control RISCome FPKM values with a cut off ≥ 1.5 . To estimate the false negative rate, results were compared by examining the number of Ago2-mRNA clusters with no predicted seeds in the top 20 Ago2-miRNAs identified by next generation sequencing. To generate Ago2-mitomiR ternary maps, we examined all mitomiR seeds in the Ago2 footprint region analysis. From these data we

looked for the greatest enrichment of miRNA seeds in the Ago2-mRNA clusters that were present within the top 20 identified miRNAs. We calculated the frequency of conserved mitomiR seed matches observed in the Ago2-mRNA footprint regions.

Cell Culture and Stable Cell Line Development

Cell culture and stable cell line development were carried out using the mouse cardiomyocyte cell line (HL-1) which maintains a cardiac-specific phenotype following repeated passaging²⁹. HL-1 cells were grown at 37°C in a humidified atmosphere of 5% CO₂ / 95% air and maintained in Claycomb media (Sigma Aldrich, St. Louis, MO) with 10% fetal bovine serum (Sigma, St. Louis, MO) and other supplements. miRNA expression plasmid (pCMV-MIR) which contains coral GFP for cellular tracking and assessment of transfection efficiency, housing the mouse precursor sequence of miR-378 (pre-miR-378) was utilized for stable cell line development (Product # sc401025; Origene, Rockville, MD).

The pre-miR-378 precursor sequence was the following: '5 -AGGGCTCCTG ACTCCAGGTC CTGTGTGTTA CCTAGAAATA GCACTGGACT TGGAGTCAGA AGGCCT-3', where the mature sequence is underlined. The pre-miR-378 sequence was situated at the 293 bp – 358 bp of a 656 bp insert. The total 656 bp sequence was situated in the multicloning site of the pCMV-MIR plasmid between Sgf I and Mlu I restriction sites.

Cells were seeded at a density of $\sim 1-3 \times 10^5$ cells/35-mm dishes and transfected at 60–70% confluence using an X-tremeGENE HP DNA Transfection Kit per the manufacturer's protocol (Roche, Indianapolis, IN). Forty-eight hours post-transfection, stable cell lines were selected in

selection media containing G418 (Gibco, Grand Island, NY). After, 4-5 passages cells were washed with phosphate-buffered saline (PBS) and harvested in either 1X RIPA Buffer (Sigma, St. Louis, MO) for total lysate or purified mitochondria isolated using a mitochondrial isolation kit (Thermo scientific, IL) for protein and enzymatic analyses.

In Vivo Antagomir Delivery in the Diabetic Heart

To determine whether manipulation of miR-378 *in vivo* influences ATP6 levels, ATP synthase functionality and ultimately, cardiac contractile function, we utilized an antagomir delivery approach. Antagomirs are anti-sense oligonucleotide sequences conjugated with cholesterol which can be delivered by injection, systemically^{30, 31}. Antagomirs provide sustained miR blockade and are currently in Phase II trials to treat a number of pathologies^{32, 33}. Linked nucleic acid (LNA) antagomirs are unique in that their sugar ring is locked with a methylene bridge placing the miR into an optimal end conformation and creating a more stable duplex leading to greater specificity for a given nucleic acid target. Four groups were utilized for the studies, 1) LNA-miR-378 (Seq: 5'TGACTCCAAGTCCAG3') (Exiqon, Woburn, MA) which was injected with STZ, 2) LNA-scrambled control (Seq: 5'ACGTCTATACGCCCA3') (Exiqon, Woburn, MA) which was injected with STZ, 3) diabetic control which was injected with STZ and 4) vehicle control (0.9% NaCl) without STZ. Five week old male FVB mice were imaged by echocardiography (described below) as a baseline after which type 1 diabetes mellitus was induced using multiple low-dose STZ injections as described above. Antagomir and vehicle treatment groups were intraperitoneally-injected once weekly for 4 weeks at a volume of 250 μ l per injection and a concentration of 25 mg antagomir/kg body weight or . This concentration and dosing schedule was chosen based on previous literature using similar LNA-miR compounds³⁴.

One week following the last injection (5 weeks total treatment time; 11 weeks of age) mice were reimaged by echocardiography. Mice were then euthanized and cardiac mitochondrial subpopulations isolated as described above, for biochemical analyses.

Cardiac Contractile Function

Transthoracic echocardiography was performed as previously described by our laboratory^{35, 36} and others³⁷. Briefly, mice were lightly anesthetized with inhalant isofluorane and transferred to dorsal recumbency. Using the Vevo 2100 Imaging System (Visual Sonics, Toronto, Canada) and a 32- to 55-MHz linear array transducer, micro-ultrasound images were acquired. M-mode images were captured via the parasternal short axis at midpapillary level with all images acquired at the highest possible frame rate (233-401 frames/s). Ejection fraction and fractional shortening were obtained from the scanned images.

Statistical Analyses

Means and standard errors (SE) were calculated for all data sets. Data were analyzed with a Student's *t*-tests along with a Welch's Correction non-parametric test (two-tailed) as determined by normality of the data (GraphPad Software Inc., La Jolla, CA). For cardiac functional parameters in the *in vivo* antagomir studies, data were analyzed with a two-way analysis of variance (ANOVA) method to evaluate the main treatment effects; therapeutic intervention and time. Tukey's post hoc tests were performed to determine significant differences among means. $P < 0.05$ was considered significant.

RESULTS

The mitochondrial proteome contains nuclear-encoded proteins that are imported from the cytoplasm as well proteins translated from its inherent genome ³⁸, which may be subject to post-transcriptional regulatory mechanisms. Recent reports demonstrate that miRNAs are associated with the mitochondrion ¹³⁻¹⁵ however subpopulation-specific miRNA profiles have yet to be examined at baseline or during pathological conditions. To determine whether type 1 diabetes mellitus influences miRNA profiles in spatially-distinct mitochondrial subpopulations, we utilized male FVB mice at five weeks post multiple low-dose streptozotocin treatment. This procedure renders mice diabetic as assessed by enhanced blood glucose levels as well as reduced plasma insulin levels ³, yet limits the toxic effects seen with a single high dose approach ^{39, 40}. We isolated and purified SSM and IFM subpopulations from diabetic and control mouse heart and confirmed purity by assessing the content by gel electrophoresis of mitochondrial specific ribosomal RNAs (16SrRNA and 12SrRNA) (Supplemental Figures S1a-b) as well as the content of the cytosolic protein, GAPDH and mitochondrial protein ATP5b (Supplemental Figure S1c).

Differential Redistribution of MitomiRs in the Diabetic Heart

We profiled the expression levels of miRNAs in diabetic and control mouse cardiac SSM and IFM using a high-throughput miRNA microarray. Profiling of the mitomiRs, revealed a significantly altered inverse expression profile following diabetes mellitus insult for many of the mitomiRs when comparing the two subpopulations. The miRNA array analyses indicated that 78 mitomiRs were differentially regulated in the two mitochondrial subpopulations following type 1

diabetic insult relative to control (Figures 4.1 and 4.2). Specifically, we observed an inverse distribution pattern between SSM and IFM following diabetic insult such that greater than 54% of identified mitomiRs were inversely distributed between the two subpopulations. These alterations in expression profiles suggest that mitomiRs may play a role in essential mitochondrial processes including the regulation of the mitochondrial genome. Confirmation of the microarray analyses was performed by selecting a mitomiR displaying significantly increased expression levels in diabetic IFM relative to control (mitomiR-378). The observed change in expression levels of this mitomiR was validated in both SSM and IFM by qRT-PCR. MitomiR-378 was differentially expressed in diabetic cardiac mitochondrial subpopulations as compared to controls (Figure 4.2) as well as in whole heart homogenate (miR-378) (Figure 4.2), confirming the microarray data.

Differential Expression of Mitochondrial Genome-Encoded Proteins in the Diabetic Heart

To determine the impact of diabetes mellitus on mitochondrial genome-encoded proteins an immunoblotting approach was undertaken to determine the subpopulation-specific expression profiles of individual mitochondrial genome-encoded proteins. Type 1 diabetic insult was associated with changes in 6 of the 13 electron transport chain proteins encoded by the mitochondrial genome. Though similar effects on spatially-distinct subpopulation protein contents were observed with diabetes mellitus (CO1, ND4), differential effects were also observed (ATP6, ATP8, Cyt B, ND6) (Figure 4.3). Of particular interest was the noted decrease in ATP6 which was observed exclusively in IFM, suggesting distinct subpopulation-specific effects on this F₀ component of the ATP synthesis machinery.

MitomiR Predicted Mitochondrial Targets and Sequence Specific Motifs

To determine whether resident mitomiRs were associated with the regulation of mitochondrial genome-encoded proteins, we utilized bioinformatic tools to predict potential mitomiR interactions with the mitochondrial genome. Using the target-prediction algorithms from various miRNA databases (RNAhybrid, MicroCosm and miRWalk), we analyzed the seed sequences from each identified mitomiR (7 seed nucleotides) and evaluated their probability of interacting with sequences from the mitochondrial genome by assessing the free energy value that characterizes the stability of the miRNA/mRNA interaction (greater than -20 kcal/mol)⁴¹. Among the 78 mitomiRs identified, 46 were predicted to bind directly to mRNA sequences from the mitochondrial genome suggesting potential regulation of translation and/or influence on mRNA stability. Using Ingenuity Pathway Analysis software to analyze mitochondrial genome targets, we observed potential interactions between all 13 mitochondrial genome-encoded electron transport chain proteins (Figure 4.4). Of the 46 mitomiRs predicted to bind to mRNA from the mitochondrial genome, 20 indicated potential binding to a single mitochondrial genome target (Figure 4.4; in red with red lines), while 26 displayed the potential to bind to multiple mitochondrial genome targets (Figure 4.4; in blue with black lines). Of interest was a predicted interaction between mitomiR-378 and ATP6 (orange line). We determined whether any specific conserved nucleotide sequence motifs existed among the 46 mitomiRs predicted to bind to mRNA from the mitochondrial genome and identified 40 mitomiRs with specific tri-nucleotide motifs suggesting sequence commonality among the majority of identified mitomiRs predicted to bind to the mitochondrial transcriptome. Specific enriched motifs included AGG, UGG and CCU tri-nucleotide sequences (Figure 4.4). Of particular interest was the observed high enrichment of UGG and AGG motifs in the 7 nucleotide seeding region (Figure 4.4). We

speculate that the existence of these motifs in a given miRNA may be predictive of its potential for distribution in the mitochondrion. Indeed, a similar type of phenomenon based upon the possession of specific sequence elements has been reported for the subcellular localization of miRNAs into other organelles ⁴².

Functional Consequences of MitomiR-378 Targeting to the Mitochondrial Genome

Because it displayed a robust increase in diabetic IFM relative to control IFM and was identified as a potential regulator of the ATP synthase, we performed additional analyses on mitomiR-378. miR-378 is of nuclear origin and originates from the first intron between exons 1 and 2 of the PPARGC1 β (PGC-1 β) gene which generates a hairpin structure (Figure 4.5) ⁴³. In the type 1 diabetic heart PGC-1 α mRNA expression was significantly down-regulated while PGC-1 β remained unchanged (Figure 4.6). These results are in agreement with a previous report ¹⁸. Bioinformatic analyses indicated that mitomiR-378 was predicted to bind to the mRNA sequence of ATP synthase F0 subunit 6 (ATP6), a constituent of the F0 component of the ATP synthase, which plays a role in the translocation of protons across in the inner mitochondrial membrane. Interestingly, mitomiR-378 was indicated as potentially binding to three different regions of the mitochondrial genome at starting positions 8,310, 8,383 and 8,515 on the ATP6 mRNA with high affinity based upon the minimum free energy (MFE) value characterizing the interaction between miRNA and target (Figure 4.6). Western blot analyses indicated significant decreases in diabetic IFM of ATP6 relative to control IFM (Figures 4.3 and 4.6). Evaluation of ATP synthase activities indicated a significant decrease in diabetic IFM with no significant effect to diabetic SSM, relative to respective controls (Figure 4.6). These data support the hypothesis that enhanced mitomiR-378 expression in diabetic IFM may account for decreases in F0 ATP

synthase subunits which lead ultimately to a decreased ability to drive the ATP synthase and generate ATP for contractile purposes.

Redistribution of RISC Components in the Mitochondrion

Small RNAs can interact with the RISC which contains proteins that include Dicer, Argonautes, and FXR1, as well as other proteins and cellular factors^{44, 45}. Assembly of the RISC and its subsequent association with a miRNA has been implicated in its biological function including regulation of mRNA expression^{46, 47}. MiRNA-mediated inhibition of translation depends largely upon the degree of complementarity between a specific miRNA or siRNA (exogenous mRNA regulators) and its mRNA target⁴⁸. RISC components such as FXR1 and Argonautes such as Ago2 interact with miRNAs during pathological conditions suggesting that stress-induced activation of the RISC contributes to its assembly⁴⁹. Examination of RISC components Ago2 and FXR1 in whole heart homogenate revealed no significant differences during diabetic insult indicating that the absolute levels of these proteins are not changed as a result of the pathology (Figures 4.7a-c). Interestingly, examination of Ago2 and FXR1 protein contents in the mitochondrion revealed that both proteins were distributed within the two subpopulations (Figures 4.8a-d). Following diabetic insult, both proteins displayed redistributed patterns with Ago2 levels decreasing in both subpopulations and FXR1 levels decreasing only in the SSM (Figures 4.8a-d). In contrast, FXR1 protein contents were significantly increased in diabetic IFM (Figures 4.8b and 4.8d). Taken together, these results indicate that RISC components Ago2 and FXR1 are present in both mitochondrial subpopulations. Further, following diabetic insult, both proteins display subpopulation-specific differential distribution patterns.

MitomiR and RISC Constituent Interactions Influence Mitochondrial Genome-Encoded Protein Abundance

Based on our broad-scale mitomiR analyses, we determined how a particular mitomiR and mRNA target interact. Chi et al. identified Ago2-miRNA-mRNA complexing using high-throughput sequencing of RNAs isolated by cross-linked immunoprecipitation (HITS-CLIP) technology in mouse brain ²⁸. Using a similar approach we determined the endogenous interactions between mitomiRs, mRNAs and mitochondrial RISC components Ago2 and FXR1 in control and diabetic mitochondrial subpopulations. Cross-linked immunoprecipitation (CLIP) was utilized to determine the presence of mitomiRs and mRNAs enriched with RISC components Ago2 and FXR1 in an effort to identify and detail the presence of a functional regulatory complex in the mitochondrion (Figure 4.9). Ago2-RNA and FXR1-RNA complexes were identified in both control and diabetic states and displayed molecular weight range shifts from 85 kDa to between 95 and 110 kDa indicating binding to the mitomiR-mRNA complex (Figure 4.10). Pull down with IgG followed by immunoblotting for biotin revealed a band in the IgG range (40-60 kDa) without bands in the above indicating specificity of the CLIP-Ago2 and CLIP-FXR-1 binding. To confirm the interaction between RISC components Ago2 and FXR1, the complex was treated with high RNase I and both RISC components immunoprecipitated with one another revealing interaction between the two proteins which was altered by the diabetic phenotype. Specifically, diabetic IFM displayed an increased interaction between Ago2 and FXR1 relative to control in both immunoprecipitation experiments which was not observed in diabetic SSM (Figure 4.10b). To determine the specific mitomiR and mRNAs present in the CLIP, portions of the membrane in which the protein-RNA complex resided were isolated and

RNA extracted after which the reverse transcribed product was utilized for cDNA synthesis. cDNA libraries were analyzed to identify enriched quantities of mitomiR-378 by qRT-PCR analysis and its association with both Ago2 and FXR1. These analyses indicated that in diabetic IFM, both RISC constituents Ago2 and FXR1 were highly associated with mitomiR-378 (Figure 4.11a). In contrast, SSM diabetic displayed little association between mitomiR-378 and Ago2 yet a strong association between mitomiR-378 and FXR1 (Figure 4.11a). These observations indicate that in the diabetic IFM, mitomiR-378 is highly associated with components Ago2 and FXR1 from the mitochondrial RISC. In contrast, diabetic SSM display low enrichment of mitomiR-378 such that the association of Ago2 with this mitomiR is limited despite the presence of FXR1. These data suggest that the regulation of mitochondrial genome-encoded proteins by mitomiRs requires the presence of a given mitomiR and the appropriate amount of mitochondrial RISC proteins Ago2 and FXR1. In the absence of either protein and/or the mitomiR, regulation of mitochondrial gene expression by the mitomiR may be limited. Complementary analyses were carried out by examining the interaction between RISC constituents Ago2 and FXR1 with mitochondrially-encoded ATP6 mRNA using qRT-PCR (Figure 4.11b). The analyses indicated that mitochondrially-encoded ATP6 mRNA was enriched in the diabetic IFM relative to control IFM mitoRISCome regardless of whether immunoprecipitating with Ago2 or FXR1. In contrast this phenomenon was not apparent in the diabetic SSM which displayed decreases in ATP6 mRNA in both Ago2 and FXR1 immunoprecipitated mitoRISComes (Figure 4.11b). These data confirm that mitochondrial RISC constituent Ago2 interacts in a ternary complex with both the specific mitomiR (mitomiR-378) and targeted mitochondrial genome mRNA (ATP6) providing a regulatory platform for the control of mitochondrial gene translation.

Next Generation Sequencing of Mitochondrial RISCome Association with Mitochondrial mRNA

Our assessment of RISC component associations with mitochondrial miRNA and mitochondria mRNA revealed differential binding patterns with Ago2 such that functional association was lost in the complex with diabetic SSM, while a strong association existed with the diabetic IFM. Thus, our analyses suggested that Ago2 presence was requisite for translational control of mitochondrial genome protein expression via mitomiR regulatory mechanisms. To capture the distinct features of the mitochondrial transcriptome functional complex, we assessed CLIP-Ago2 (mitoRISCome)/mRNA complex products utilizing true RNA-seq library preparations to establish mitochondrial mRNA transcripts through sequencing with a high-throughput Mi-seq platform. Alignment of sequenced reads to the mouse mitochondrial genome, revealed that in the non-diabetic condition, differential proportions of the mitochondrial transcriptome (mitochondrial mRNA) were associated with the mitoRISCome in SSM (69%) and IFM (31%). However, these percent associations were altered with diabetes mellitus treatment differentially in SSM and IFM such that a greater portion of the IFM transcriptome (49%) was associated with the mitoRISCome, while a lesser portion of the SSM transcriptome (51%) was associated with the mitoRISCome (Figure 4.12a and 4.12b). Specific associations between RISC component (Ago2) and each of the 13 mitochondrially-encoded proteins under control and diabetic conditions are indicated in Figure 4.13. The presence of mitochondrial mRNAs in the different groups varied depending upon treatment and subpopulation type. Of particular interest was the presence of mRNA encoding ATP6 in the mitoRISCome of diabetic IFM which was not present in the control IFM (blue box). This finding was confirmatory of those in the mitoRISCome analyses (Figure 4.11b). Our analyses identified 9 mitochondrially-encoded

mRNAs in the diabetic subpopulations which were differentially expressed in the mitoRISCome relative to controls (Figure 4.14a). Further, 6 of the differentially expressed mRNAs displayed opposite expression patterns from their subpopulation counterpart (Figure 4.14a). Mapping of the mitochondrial mRNAs identified in the mitoRISCome next generation sequencing enabled identification of specific cluster regions in each of the 13 mRNA transcripts which were present in the mitoRISCome (Figure 4.14b; blocks indicated in red). To gain insight into the relationship between these cluster sequences and mRNA binding sites, we performed an unbiased search of associated nucleotide sequences in the identified clusters to determine whether specific sequence motifs were common within the identified clusters. Our results indicated that these clusters were enriched with a UCC motif in all 9 mitochondrial transcripts identified, and this motif was highly enriched in the ATP6 transcript (Figures 4.15a and 4.15b).

Next Generation Sequencing of Mitochondrial RISCome Association with MitomiRs

To capture the distinct features of the mitomiR signatures in the mitoRISCome functional complex we assessed mitoRISCome/mitomiR complex products utilizing small RNA-seq library preparation to establish mitomiR transcripts through sequencing with a high-throughput Mi-seq platform. High throughput sequencing of small RNAs in the mitoRISCome identified 37 mitomiRs which were differentially regulated as a result of diabetic insult in mitochondrial subpopulations (Supplementary Figure 2). Of the 37 mitomiRs identified, 10 (Figure 4.16a) were also observed in our initial microarray experiments (Figure 4.1) and underwent prediction analyses (Figure 4.4a). Interestingly, our high throughput sequencing of small RNAs in the mitoRISCome revealed the presence of a number of mitomiRs that were not identified in our initial microarray analyses. Several variables may have accounted for the discrepancy including

the lack of miRNA probes on the microarray (miRNA- 3963, -5102, -5105, -5108, -5109, -5112, -5115, -5119, and -5131). Thus, our microarray data potentially underestimate the absolute number of mitomiRs. As microarray technologies continue to advance, it is probable that a greater number will be identified in the mitochondrion in the future. Further, studies suggest comparing results from microarray and next generation sequencing shows good correlation between expression levels for a given gene between the two platforms, and the use of both can provide complementary information. However, issues such as sensitivity, background noise interference and hybridization saturation can lead to differences in results ⁵⁰. Among the identified mitomiRs in the mitoRISCome was mitomiR-378 (Figure 4.16a). Of particular interest was the observation that the microarray and snapshot mitoRISCome analyses, though identifying similar mitomiRs, revealed differential signature patterns, such that the relative ratio of mitomiR in the mitochondrion (Figure 4.1) did not necessarily match the data from the mitoRISCome analyses (Figure 4.16a). These data suggest that the presence of the mitomiR in the mitochondrion does not necessarily reflect it as possessing a functionally active regulatory role on the mitochondrial transcriptome at a given time. Such a phenomenon is in agreement with other studies ^{51, 52}. Moreover, our current study revealed that functional miRNA:mRNA interactions are not limited to the regulation of nuclear-encoded genes, but may also play a role in the regulation of mitochondrially-encoded genes. Diabetic IFM displayed the greatest response in terms of increased functional mitomiRs relative to control (Figure 4.16a). Mapping of the mitomiRs identified in the mitoRISCome next generation sequencing which were also identified in the microarray analyses enabled identification of specific regions in each of the 10 mitomiRs present in the mitoRISCome (Figure 4.16b). As with the mRNA analyses, we performed an unbiased search of associated sequences in the 3-22 nucleotide regions of these 10

mitomiRs to determine whether specific sequence motifs were common. Our results revealed enriched nucleotide motifs, AGG (in red) and UGG (in yellow) in all 10 mitomiRs, and these motifs were highly enriched in mitomiR-378 (Figures 4.16b and 4.16c). To confirm the presence of mitomiR-378 in the mitoRISCome we determined whether the mature mitomiR-378 was present. Mapping of the next generation sequencing indicated the presence of mitomiR-378 solely in the diabetic IFM of which only the mature transcript was apparent (Figure 4.17a). Sequence scanning analysis of the ternary complex revealed mitomiR-378 binding sites were present within the footprint region of the ATP6 transcriptome at starting positions 8,310 with a secondary binding site at position 8,383 (Figure 4.17b). These results were consistent with the previous bioinformatically-predicted targets for mitomiR-378 (Figure 4.6a).

miR-378 Overexpression in a Cellular System

To validate the regulatory activity of mitomiR-378 on ATP6, we generated a stable HL-1 cell line overexpressing the mouse miR-378 which contained a GFP coding sequence to enable cellular tracking. To determine whether down-regulation of ATP6 and ATP synthase functional decrements were associated with miR-378 presence in the mitochondrion, mitochondria was isolated from miR-378 overexpressing HL-1 cells and the presence of mitomiR-378 examined by qRT-PCR analyses. Our data revealed an approximately 22 fold increase of mitomiR-378 in isolated mitochondria from the stable HL-1 cell line overexpressing miR-378 as compared to control HL-1 cells (Figure 4.18a). Western blot analyses revealed a significant decrease in the expression of ATP6 in the mitochondrion (Figures 4.18b and 4.19b) with a corresponding decrease in ATP synthase activity (Figure 4.19a). Taken together, these data provide complementary evidence that in a model of enhanced miR-378 expression, translocation to the

mitochondrion can occur which is associated with a decrease in mitochondrial genome-encoded ATP6 and a concomitant reduction in the functionality of the ATP synthase.

Manipulation of miR-378 In Vivo with Targeted Antagomir Delivery

To determine whether manipulation of miR-378 *in vivo* could influence ATP generating capacity through ATP6 preservation, we utilized an LNA-linked antagomiR delivery approach targeting miR-378 (LNA-miR-378). Repeated injection of LNA-miR-378 over a five week period led to preservation of ATP6 protein levels in diabetic IFM which were similar to non-diabetic controls and significantly higher than diabetic and scrambled miR diabetic groups (Figure 4.20b). In contrast, no significant differences in ATP6 protein levels were observed among any group in the SSM (Figure 4.20a). To determine whether preservation of ATP6 protein levels impacted ATP generating capacity, we assessed ATP synthase activities following LNA-miR-378 intervention and diabetes mellitus. As with ATP6 protein levels, ATP synthase activities displayed similar changes in diabetic IFM, with LNA-miR-378 treatment providing restoration of ATP synthase activity which was significantly decreased in diabetic and scrambled miR diabetic groups (Figure 4.20d). Further, no significant differences in ATP synthase activities were observed among any group in the SSM (Figure 4.20c). Because preservation of ATP synthase activity ultimately influences cardiac pump function by providing necessary ATP levels, we assessed ejection fraction and fractional shortening changes prior to and following LNA-miR-378 intervention and diabetes mellitus. Our results revealed preservation of %ejection fraction (Figures 4.21a and 4.21b) and %fractional shortening (Figure 4.21a and 4.21c) in LNA-miR-378 diabetic hearts which was significantly decreased in diabetic and scrambled miR diabetic hearts. Taken together, these findings provide *in vivo* confirmation of an LNA-miR-378

impact on diabetic IFM which manifests as preservation of ATP6 protein content, ATP synthase levels and cardiac pump function.

DISCUSSION

Our findings indicate for the first time that mitomiRs are differentially distributed in spatially-distinct cardiac mitochondrial subpopulations. Upon diabetic insult, cardiac mitomiR distribution patterns are dramatically altered and in many cases display opposite/reciprocal arrangements. Type 1 diabetes mellitus is associated with cardiac contractile deficits, which may result from a decreased ability for cardiac mitochondria to generate ATP. Among the genes encoded for by the mitochondrial genome are 13 proteins of the electron transport chain that are constituents of complexes I, III, IV, and V. Thus, regulation of the mitochondrial genome by mitomiRs may have profound effects on the expression of key components driving ATP synthesis and ultimately influencing cardiac contractile function.

Using a number of bioinformatics tools, we identified 46 mitomiRs that were predicted to bind to mitochondrial genome-encoded mRNAs. Over half of the identified mitomiRs displayed the potential for binding multiple mitochondrial-encoded genome mRNAs. Such a finding is intriguing and suggests the potential for an individual mitomiR to regulate the translation of multiple mitochondrial genome products. Such a dynamic is consistent with proposed mechanisms of action for other miRNAs^{53, 54} and as a result mitomiR manipulation for therapeutic intervention represents an attractive strategy for addressing pathological conditions, such as diabetes mellitus, as it may offer the ability to rectify proteomic loss of multiple mitochondrial genome-encoded proteins simultaneously. Additional bioinformatic analyses of the nucleotide sequences in the 46 identified mitomiRs indicated that ~ 85% possessed specific conserved tri-nucleotide motifs which included AGG, UGG, and CCU. Of particular interest

was an observed concentration of AGG and UGG motifs contained in the 2-7 nucleotide seeding region of a number of mitomiRs. We speculate that the existence of these motifs in the mitomiR may influence its translocation into mitochondria and that their presence may predict the potential for a given miRNA to exist in the mitochondrion. Further investigation is needed to determine the relative importance of these particular motifs.

Because it displayed a dynamic change in its content following diabetic insult and a reciprocal response in spatially-distinct mitochondrial subpopulations, we chose to examine mitomiR-378 for our subsequent studies. miR-378 plays an important regulatory role in cardiac remodeling, cell survival^{55, 56} and its complimentary pair, miR-378*, mediates a metabolic shift to oxidative phosphorylation^{18, 43}. Though it displayed a significant increase in the heart following diabetic insult, miR-378 was dramatically increased only in the IFM suggesting that its enhanced presence may be of particular relevance specifically to the mitochondrion. Identification of a mitochondrial genome target for mitomiR-378, ATP6 and the associated decrease in ATP synthase activity specifically in the IFM, suggest a coordinated regulatory role for mitomiR-378 in type 1 diabetic IFM. Manipulation of this axis may contribute to the development of diabetic cardiomyopathy by compromising the generation of ATP. Indeed, overexpression of miR-378 in a cell type that maintains a cardiac phenotype revealed increased mitochondrial presence and a concomitant decrease in ATP6 content and function in an *in vitro* model. Further, utilization of an antagomir approach directed at miR-378 demonstrated down-regulation of ATP6, ATP synthase activity, and cardiac pump function confirming this axis *in vivo*. Interestingly, examination of the type 2 diabetic (*db/db*) heart revealed a similar increase in mitomiR-378 presence in the SSM which was associated with its increased presence in the SSM

mitoRISCome (Supplementary Figure 3a-b). Though *db/db* IFM also displayed an increase in mitomiR-378 presence, no significant increase in the *db/db* IFM mitoRISCome was observed (Supplementary Figure 3a-b), suggesting lack of a functional interaction with the mitochondrial genome in this subpopulation. These findings are in agreement with our previous study revealing mitochondrial dysfunction in the *db/db* heart which was confined primarily to the SSM subpopulation²⁵. Taken together our findings suggest enhanced mitomiR-378 presence in the type 1 diabetic IFM and type 2 diabetic SSM, the mitochondrial subpopulations most impacted by their associated pathology, may be a common mechanism contributing to bioenergetic deficits occurring in the face of the diabetic phenotype. Additional comprehensive studies in the type 2 diabetic heart are warranted.

Perhaps the most interesting finding in the current study was the identification of RISC presence in the mitochondrion which contained key components, Ago2 and FXR1. It appears that the mitochondrial RISC functions similarly to its cytoplasmic counterpart, influencing translation. Reports of changes in miRNA expression, has provided indirect evidence for translational repression, yet evidence for a direct effect of the miRNA on the mRNA is limited. Use of the CLIP experimental approach provides a clear means of identifying direct miRNA/mRNA/protein interactions confirming direct association between nucleotide and protein components²⁸. Following type 1 diabetes mellitus, mitochondrial RISC components were differentially expressed in the mitochondrion relative to control and the response was subpopulation-specific in nature. Our results indicate that translational regulation of ATP6 of the mitochondrial genome by mitomiR-378 requires both Ago2 and FXR1 presence in the RISC, as well as the necessary mitomiR (mitomiR-378) as observed in diabetic IFM. When Ago2 content

is limiting and the mitomiR (mitomiR-378) is not present, regulation via the mitochondrial RISC does not occur, as observed in diabetic SSM (Figure 4.22). Our results are in agreement with literature suggesting the relative importance of FXR1 presence in that its knockout is lethal in mice and in zebrafish as a result of cardiomyopathy and muscular dystrophy⁵⁷. Reports indicate a requirement of both FXR1 and Ago2 in the RISC for translational activation^{12, 58-60}, via AU-rich elements. Our current data extend these findings by identifying a similar phenomenon within the mitochondrion that occurs in a subpopulation-specific manner during diabetic insult and requires specific sequence motifs (AGG and UGG) in the miRNA sequence. Thus, redistribution of mitomiRs and the presence of specific mitochondrial RISC component stoichiometry, contributes to the formation of a functional complex which is associated with loss of mitochondrially-encoded proteins in the diabetic heart.

Recent crystallographic and NMR structural analyses have indicated that all three molecules (mRNA, miRNA and protein) require close proximity and direct interaction to facilitate a functional cytoplasmic ternary regulatory complex^{28, 51, 61-63}. Nevertheless, identification of a functional RISC in the mitochondrion is highly challenging. Recent reports suggest that RNA can be imported into the mitochondrion^{64, 65} which supports our current findings. The use of CLIP technology coupled with next generation sequencing offers an experimental platform for determining binding maps and sequence motifs necessary for miRNAs to exert regulatory influence on mRNA expression/translation. Our CLIP analyses confirmed that mitomiR-378 targets two different sites on the ATP6 mRNA, which was bioinformatically predicted prior to sequencing analyses. These results were interesting in that miRNAs have been identified as binding to the 3'-untranslated region of mRNA to exert regulatory activity.

Nevertheless, the mitochondrial genome does not possess 3'-untranslated regions, rather the sequence identity from the bioinformatic and next generation sequencing analyses predicted binding directly to the coding region. Such a finding is consistent with previous studies demonstrating that the mouse *Nanog*, *Oct4* and *Sox2* genes were targeted by miRNA in their amino acid coding sequence resulting in translational repression⁶⁶. Our results indicate that mitomiR-378 targets the ATP6 coding region in a similar manner to control translational activity.

In summary, we propose that mitomiR translational regulation of mitochondrially-encoded proteins oscillates in spatially-distinct mitochondrial subpopulations in response to type 1 diabetic insult. Redistribution of mitomiRs and the presence of a specific mitochondrial RISC component stoichiometry, contributes to the formation of a functional ternary complex which is associated with loss of mitochondrially-encoded proteins in the diabetic heart. These results suggest a dynamic system that can regulate the mitochondrial proteome during pathological states in a spatially-distinct manner.

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DISCLOSURES

None

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Figure 4.1

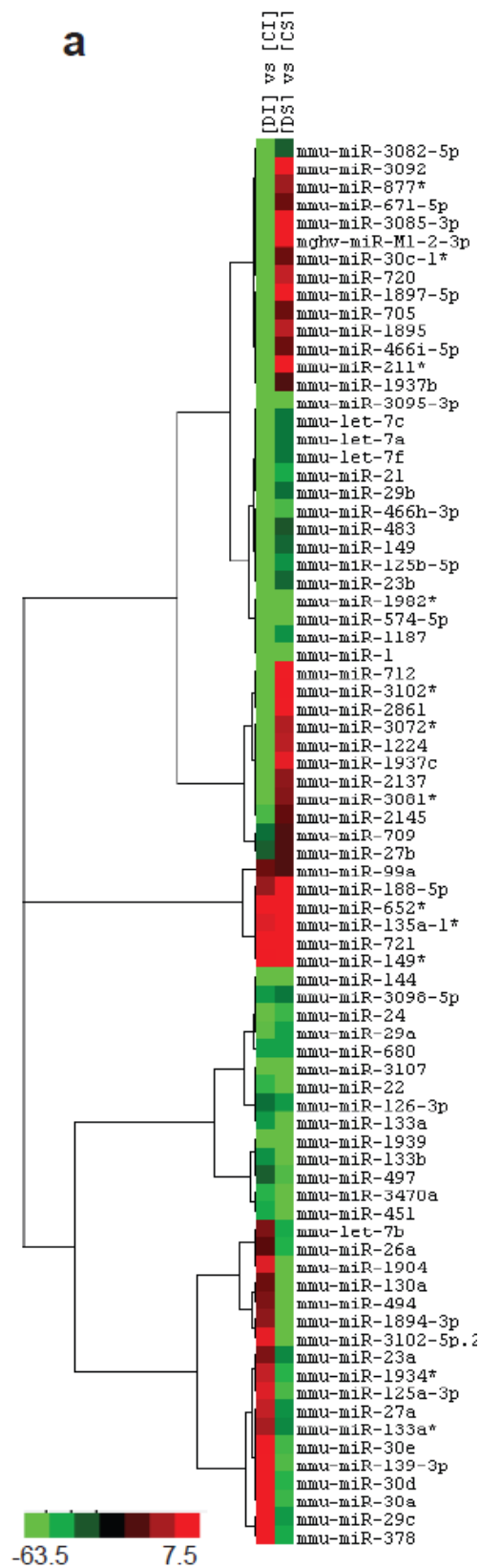
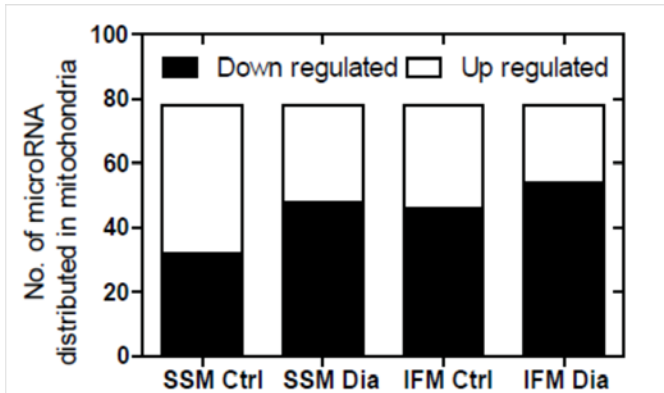


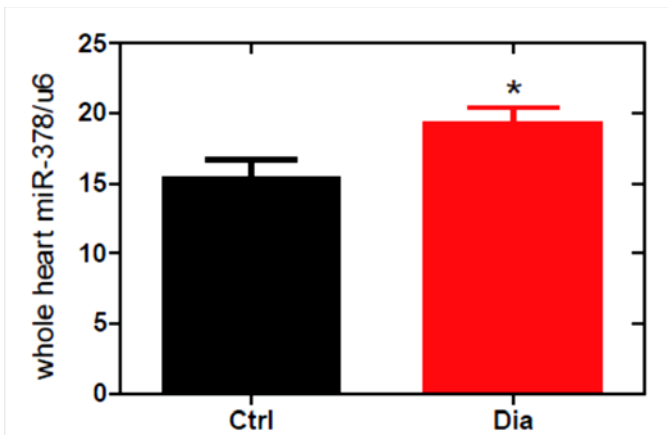
Figure 4.1. Differential mitomiR expression patterns in cardiac mitochondrial subpopulations. (a) Hierarchical clustering heat map for the microarray analysis of mitomiR expression profiles in cardiac mitochondrial subpopulations. DI = diabetic IFM, CI = control IFM, DS = diabetic SSM, CS = control SSM; n = 4 for each group. All mitomiRs reported in the heat map are significantly different relative to control.

Figure 4.2

a



b



c

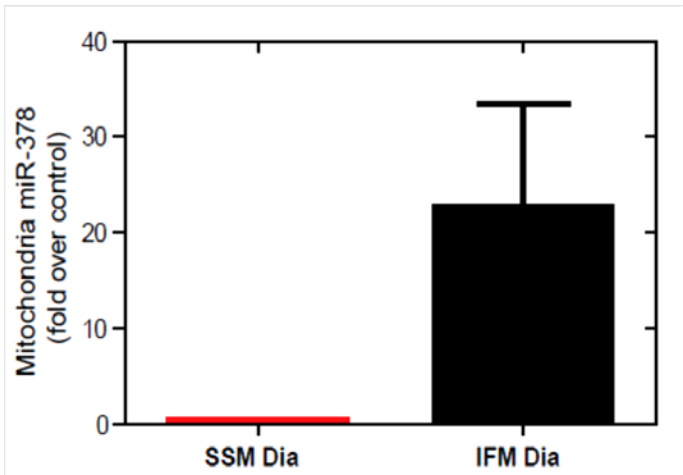


Figure 4.2. Differential mitomiR expression patterns in cardiac mitochondrial subpopulations. (a) Differential distribution (up-regulated or down regulated) patterns of mitomiRs in SSM and IFM control and diabetic mitochondrial subpopulations. (b) qRT-PCR analyses of miR-378 in control and diabetic whole heart tissue. Values are represented as mean \pm SE. * $P < 0.05$ for control vs. diabetic. U6 mRNA served as control. (c) qRT-PCR analyses of mitomiR-378 in SSM and IFM diabetic mitochondrial subpopulations as compared with control. Values are represented as mean \pm SE. U6 mRNA served as control.

Figure 4.3

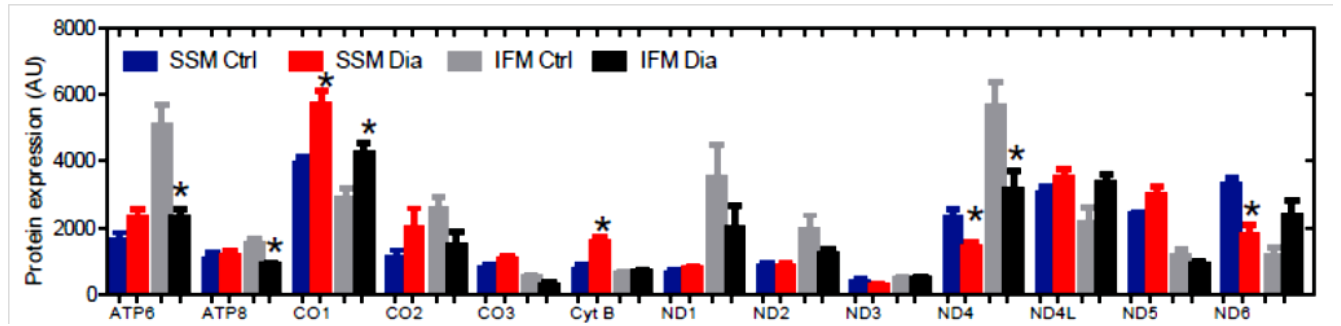


Figure 4.3. Mitochondrial genome-encoded proteins in mouse cardiac mitochondrial subpopulations. (a) Mitochondrial genome-encoded protein expression in mitochondrial subpopulations. Values are represented as mean \pm SE of SSM Control, SSM Diabetic, IFM Control, and IFM Diabetic; * $P < 0.05$.

a

[illegible]

Nucleotide region	CCU	UGG	AGG
1-3	0	1	2
2-4	1	1	0
3-5	1	7	6
4-6	1	1	2
5-7	1	0	0
6-8	0	1	1
7-9	0	1	1
8-10	1	2	1
14-16	0	1	0
16-18	0	2	2
17-19	0	1	1
19-21	1	0	0

Figure 4.4. Predicted mitomiR target networks in mouse cardiac mitochondrial subpopulations. (a) Prediction analyses for mitomiR targeting to mitochondrial genome-encoded mRNAs using bioinformatic databases RNAhybrid, miRWalk and Microcosm. Mitochondrial genes (Focus genes) were overlaid into a global mitomiR network developed from the information contained in the Ingenuity Pathways Knowledge Base. Focus gene networks were algorithmically generated based on their connectivity. The network pathway is a graphical representation of the molecular relationships between mitomiR and mitomiR or mitomiR and mitochondrial encoded gene. Those mitomiRs predicted to bind a single mitochondrial genome target are indicated in red with red lines, while those mitomiRs predicted to bind multiple mitochondrial genome targets are indicated in blue with black lines. MitomiR-378 predicted interaction with ATP6 is indicated with an orange line. (b) Conserved sequence specific trinucleotide motifs and their location within the identified mitomiRs.

Figure 4.5

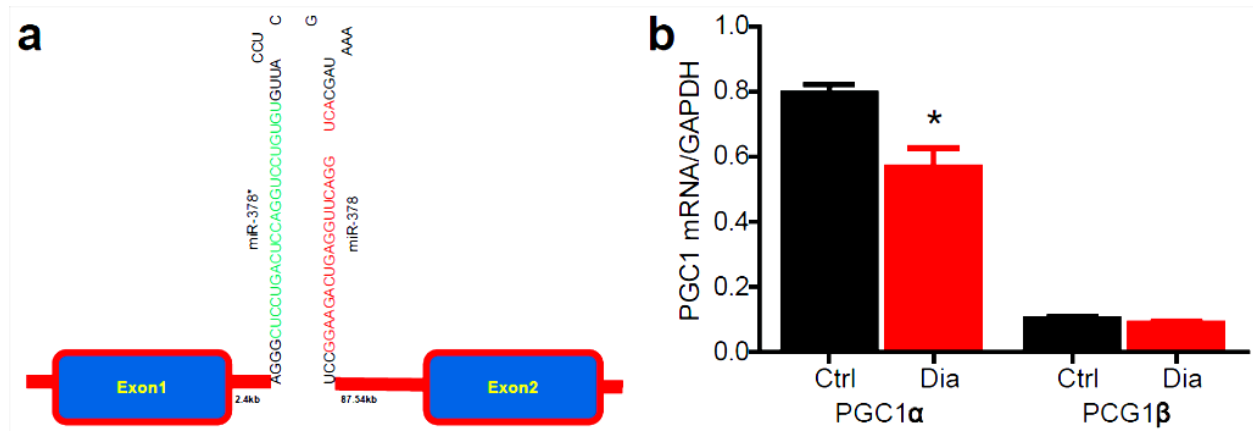
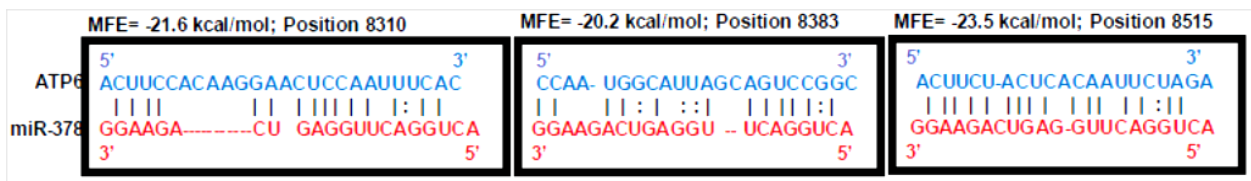


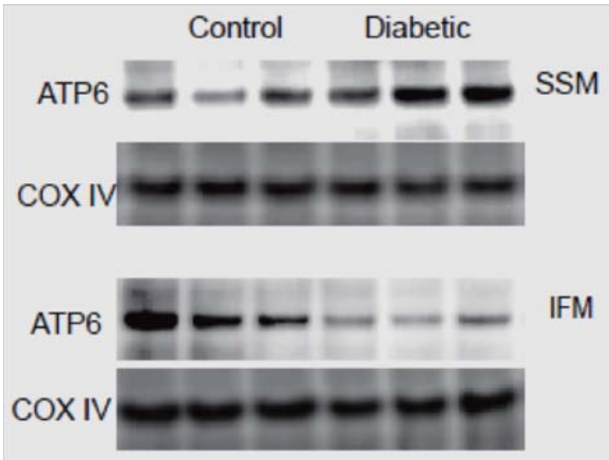
Figure 4.5. MitomiR-378 and its targeting to the mitochondrial genome. (a) Schematic representation of miR-378 location in the PPARGC1b (PGC-1b) gene. It is located in between exon 1 and exon 2. (b) qRT-PCR quantification of PGC1 α and PGC1 β transcripts in control and diabetic hearts. GAPDH served as control. Values are represented as mean \pm SE; n=4 for each group. * $P < 0.05$ for Control vs. Diabetic.

Figure 4.6

a



b



c

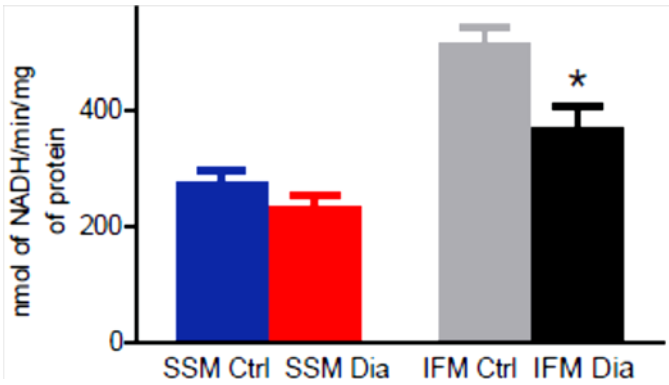
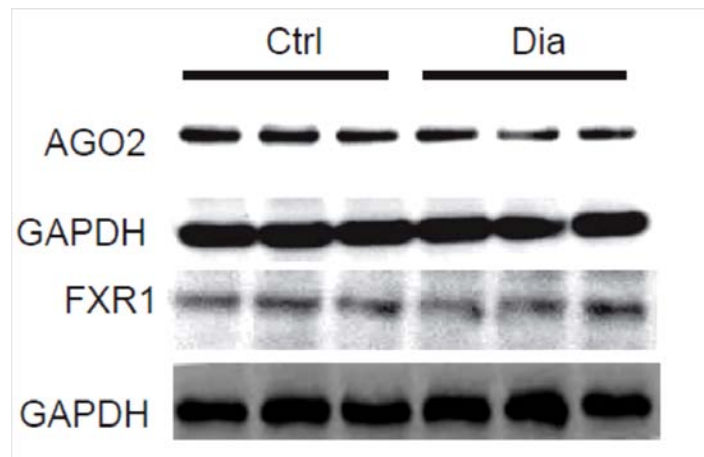


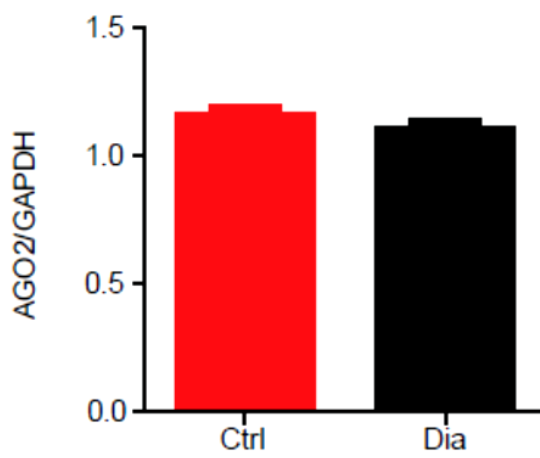
Figure 4.6. MitomiR-378 and its targeting of ATP6 and ATP6 protein content in control and diabetic subpopulations. (a) Evaluation of the minimum free energy (MFE) value that characterizes the stability of mitomiR-378/mRNA interaction, identified by MicroCosm and RNAhybrid. (b) Western blot analysis of ATP6 in control and diabetic SSM and IFM. Cox IV serves as a loading control. (c) ATP synthase activity expressed in activity/min/mg protein. Values are means \pm SE; n = 8 for each group. * $P < 0.05$ for Control IFM vs. Diabetic IFM.

Figure 4.7

a



b



c

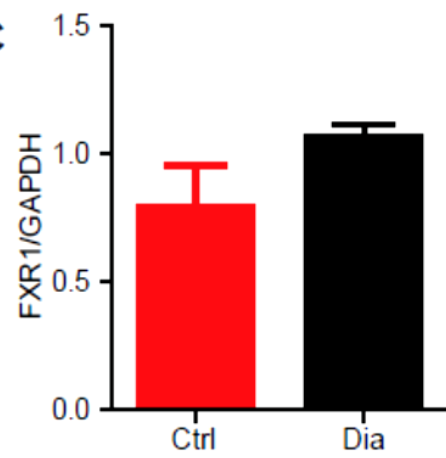


Figure 4.7. Redistribution of RISC components in control and diabetic whole heart. (a)

Western blot analyses of Ago2 and FXR1 in control and diabetic whole heart. GAPDH serves as

loading control. **(b)** Quantification of Ago2 protein content in control and diabetic whole heart.

Values are means \pm SE. **(c)** Quantification of FXR1 protein content in control and diabetic whole

heart. GAPDH served as loading control. Values are means \pm SE.

Figure 4.8

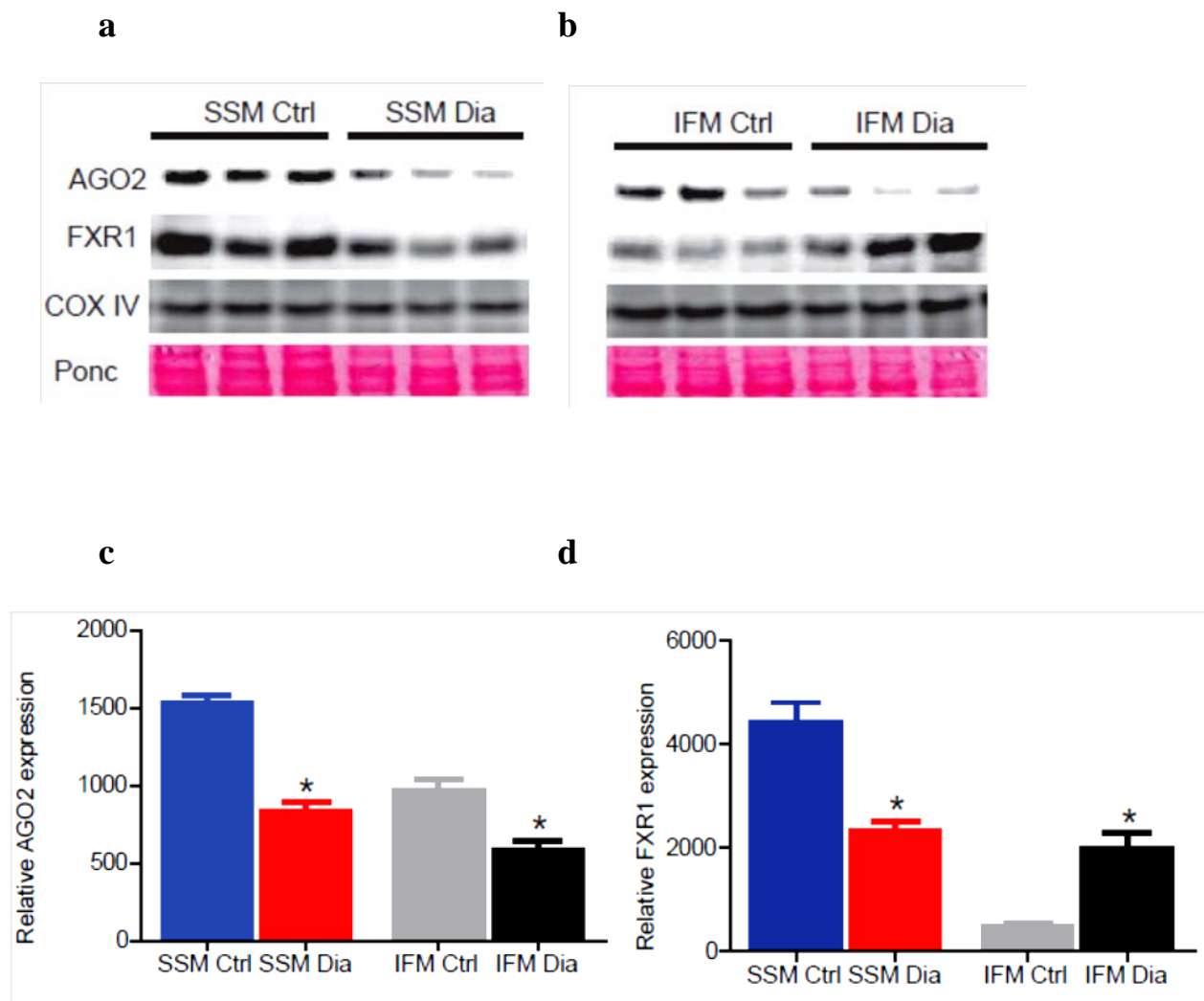


Figure 4.8. Redistribution of RISC components in control and diabetic mitochondrial subpopulations. (a) Western blots analyses of Ago2 and FXR1 in Control and Diabetic SSM. COX IV serves as a loading control. (b) Western blots analyses of Ago2 and FXR1 in Control and Diabetic IFM. COX IV serves as a loading control. (c) Quantification of Ago2 protein content in control and diabetic mitochondrial subpopulations. Values are means \pm SE. $*P < 0.05$ for Control vs. Diabetic. (d) Quantification of FXR1 protein content in control and diabetic mitochondrial subpopulations. Values are means \pm SE. $*P < 0.05$ for Control vs. Diabetic.

Figure 4.9

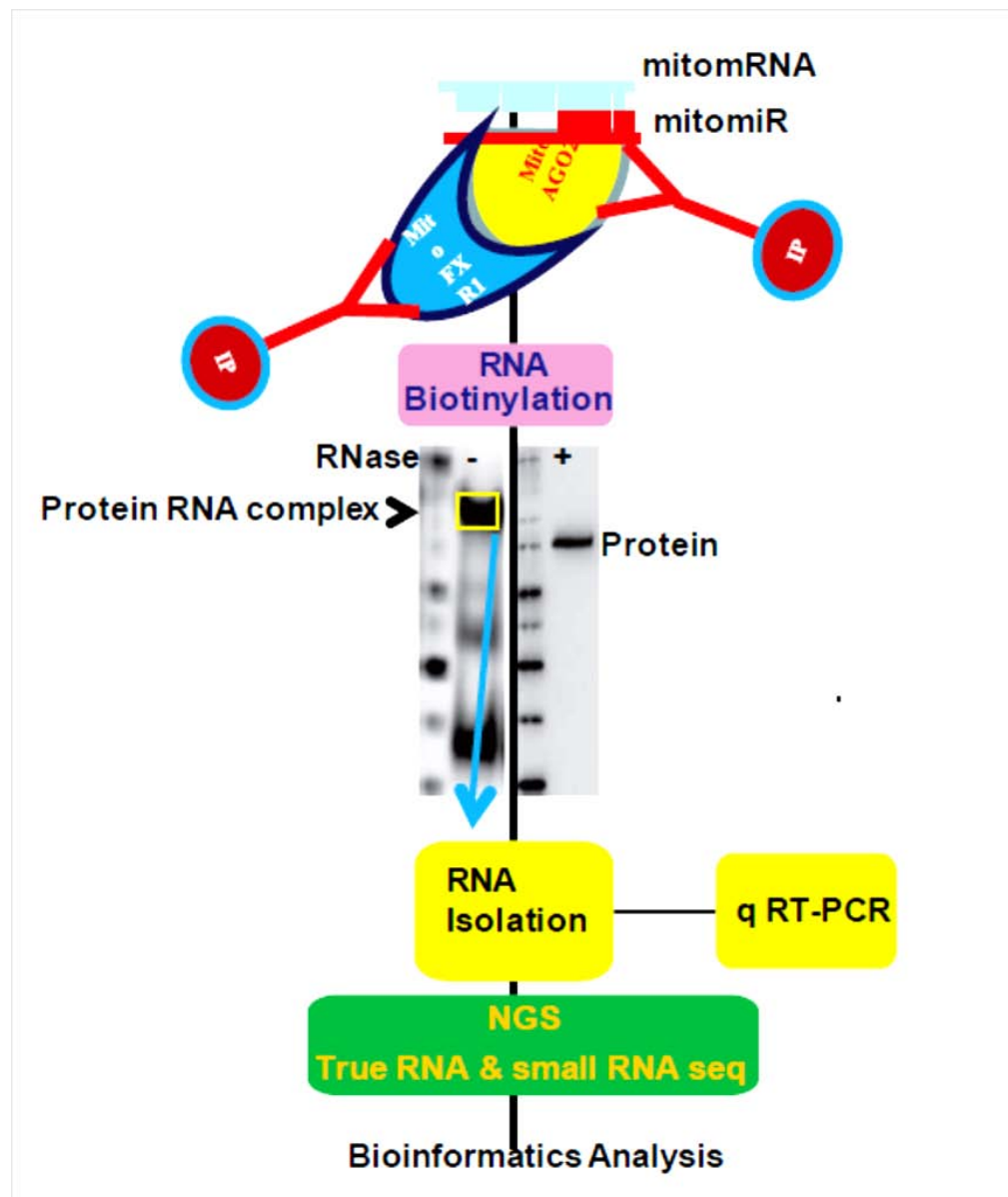


Figure 4.9. Crosslinked immunoprecipitation (CLIP) in cardiac mitochondrial subpopulations. Schematic representation of CLIP-Ago2 and the CLIP-FXR1 experimental designs. The association of mitomRNA and mitomiR requires to be in a complex with both RISC components (Ago2 and FXR1). Immunoblotting with biotin is used to pull down protein and RNA complex from both CLIP-Ago2 and CLIP-FXR1. RNA isolation is then performed which is subjected to next generation sequencing and small RNA seq.

Figure 4.10

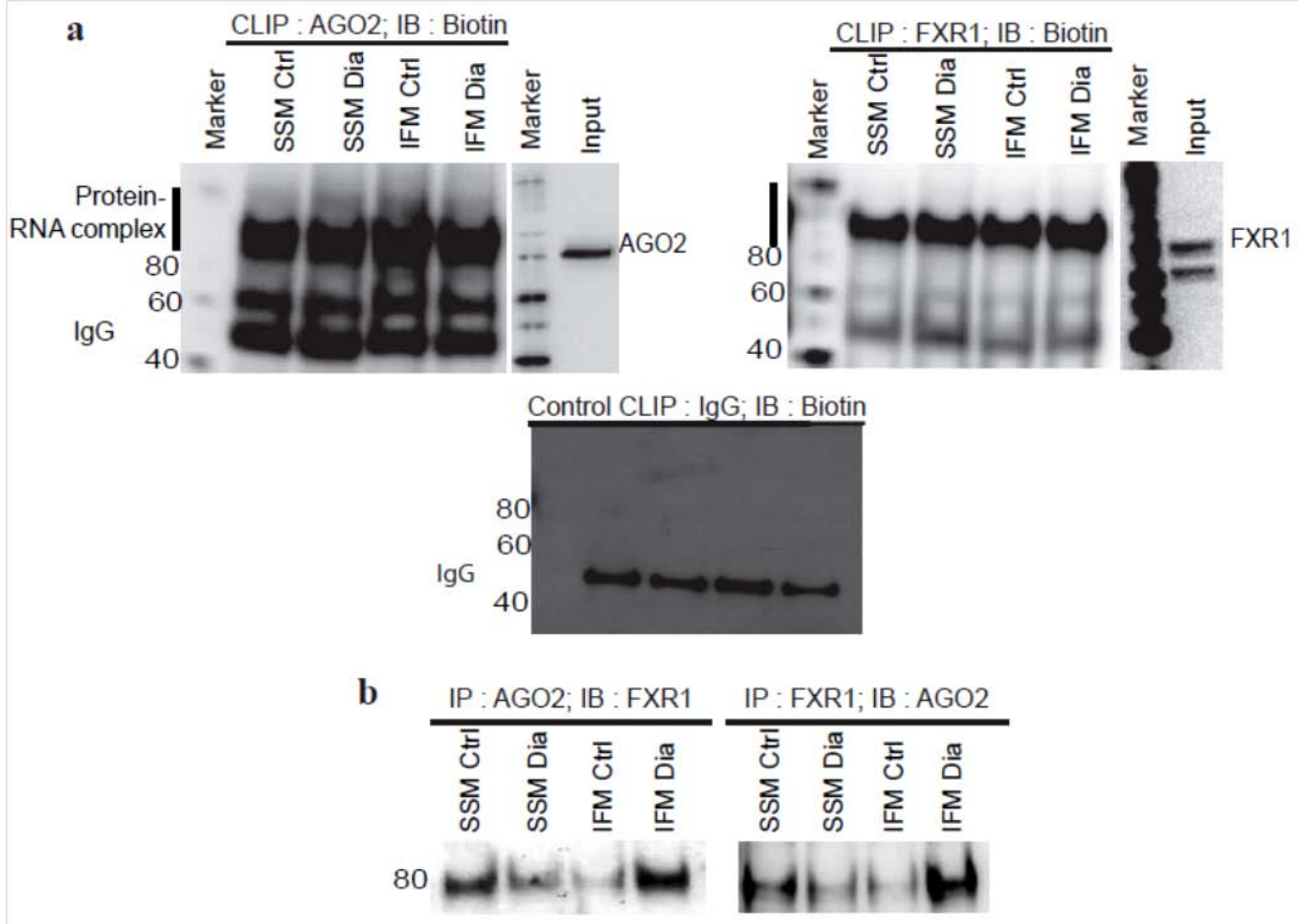


Figure 4.10. Crosslinked immunoprecipitation (CLIP) in cardiac mitochondrial subpopulations. (a) Western blots of biotinylated RNA from CLIP-Ago2 and CLIP-FXR1 reactions illustrating crosslinked protein/RNA and the associated gel shift from 80 kDa to 95-110 kDa. Pull down with IgG followed by immunoblotting for biotin revealing specificity of the CLIP-Ago2 and CLIP-FXR-1 binding. (b) Western blot analyses of CLIP-Ago2 and CLIP-FXR1 subjected to RNAase I treatment at 1:50 dilution (high RNAase) illustrating interaction between the two proteins in the absence of RNA.

Figure 4.11

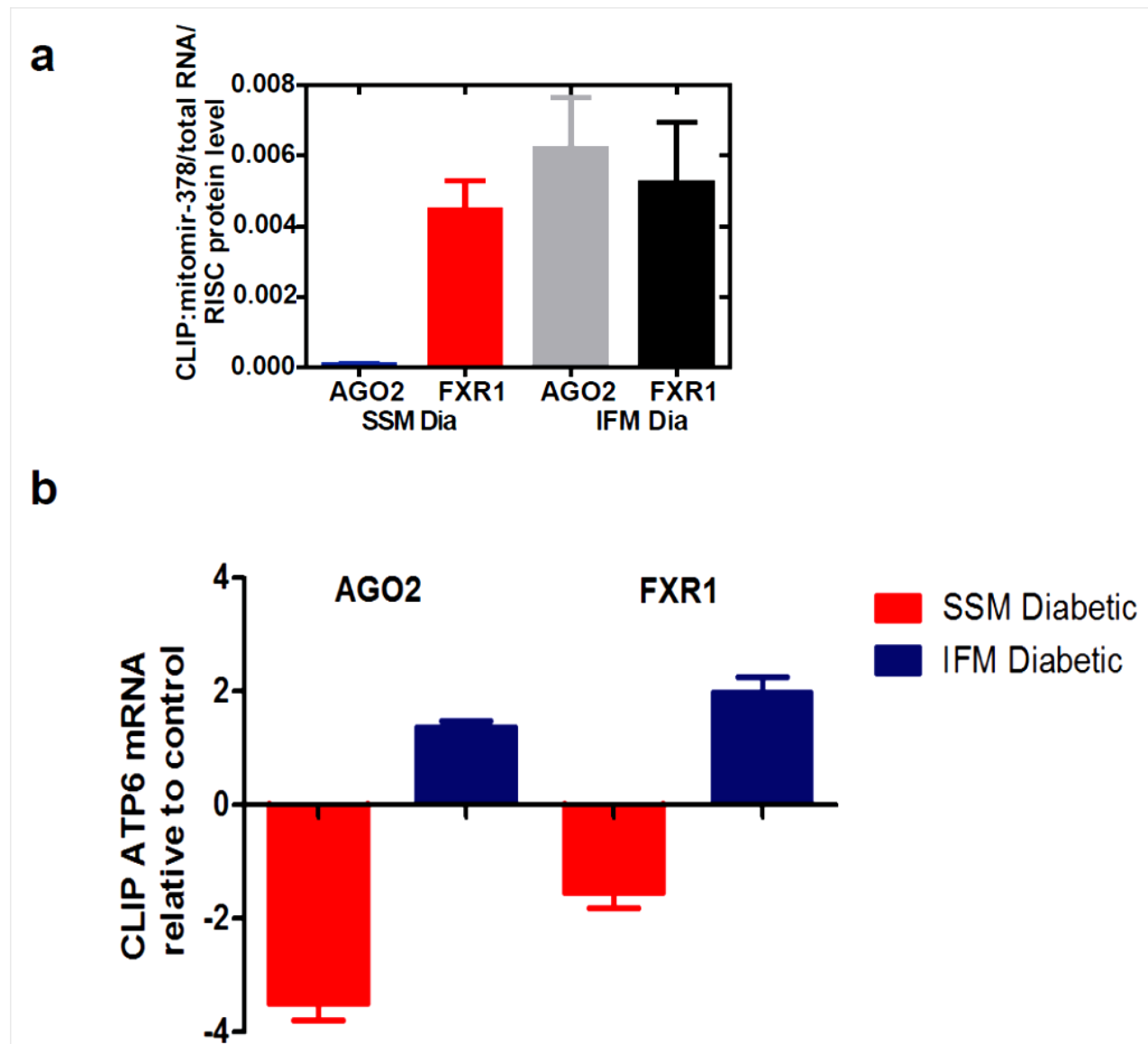


Figure 4.11. MitomiR-378 and RISC constituent interactions with the mitochondrial genome. (a) CLIP-Ago2 and CLIP-FXR1 associated enrichment analyses of mitomiR-378 analyzed by qRT-PCR in control and diabetic cardiac mitochondrial subpopulations. Values are presented as means \pm SE. (b) CLIP-Ago2 and CLIP-FXR1 associated enrichment analysis of transcripts for mitochondrial encoded ATP6 mRNA levels as assessed by qRT-PCR analysis in control and diabetic cardiac mitochondrial subpopulations. Values are presented as means \pm SE.

Figure 4.12

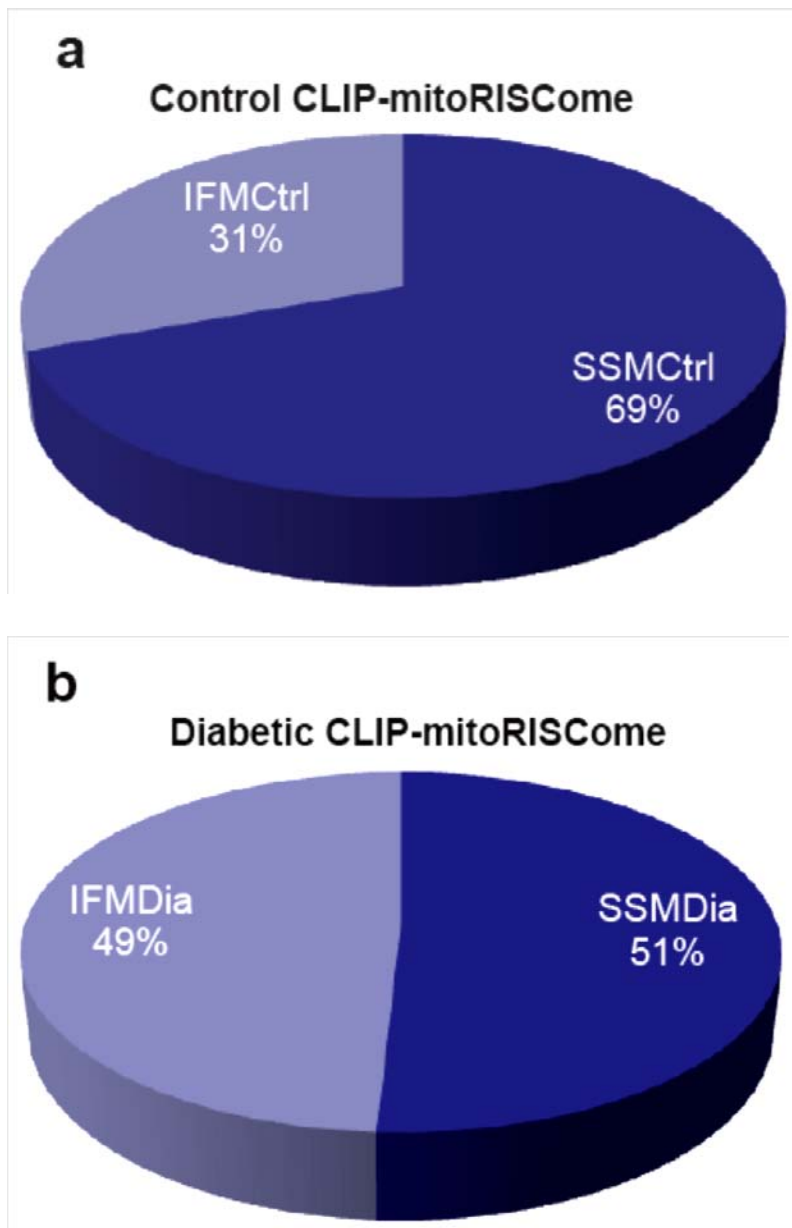


Figure 4.12. Genomic sequencing analyses of mitochondrial RISCome association with mitochondrial mRNA. Proportion of RNA-seq reads mapping to the mitochondrial transcriptome (mitochondrial mRNA) of mitochondrial subpopulations in **(a)** control and following **(b)** diabetic insult.

Figure 4.13

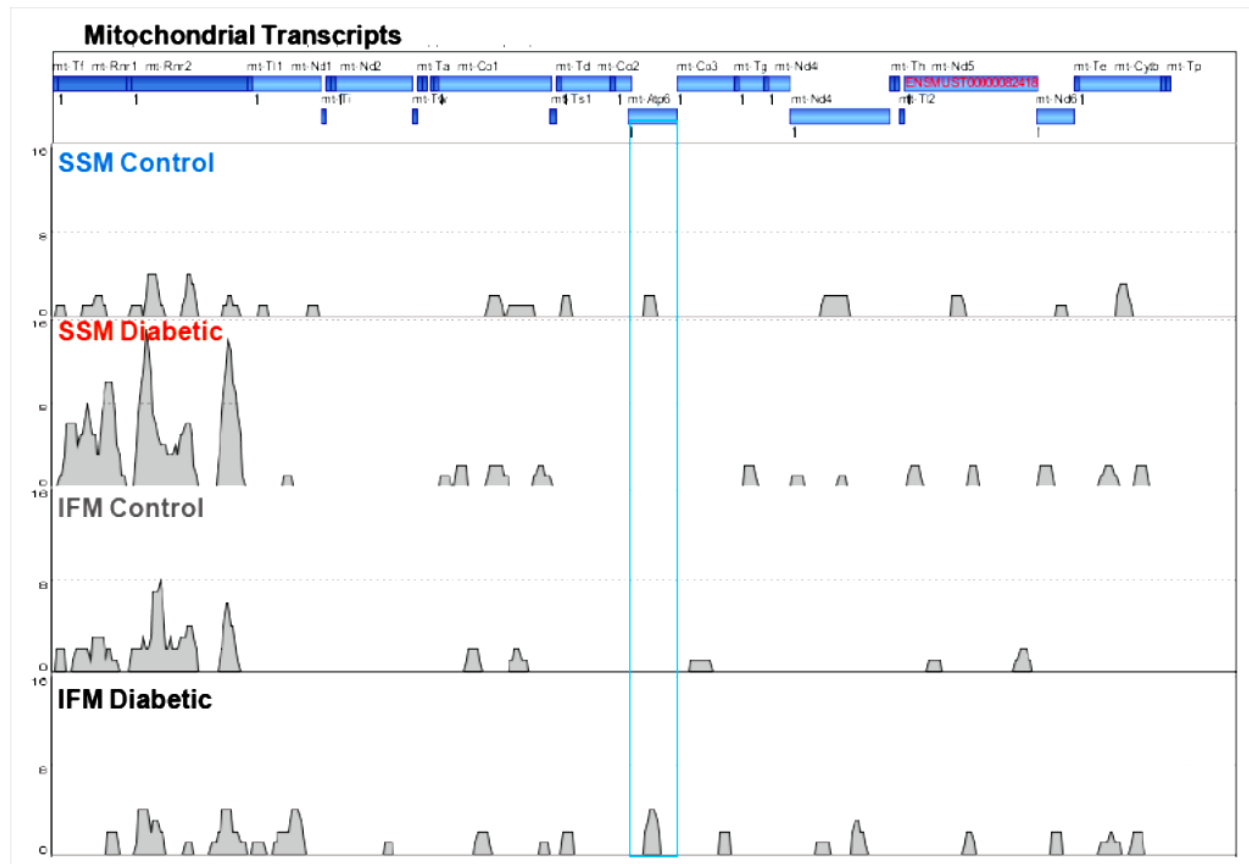


Figure 4.13. Genomic sequencing analyses of mitochondrial RISCome association with mitochondrial mRNA. Genome browser illustration of mitochondrial RNA-seq reads mapping to rRNA, tRNA, mRNAs; histogram indicates RNA-seq read distribution which includes the transcript region.

Figure 4.14

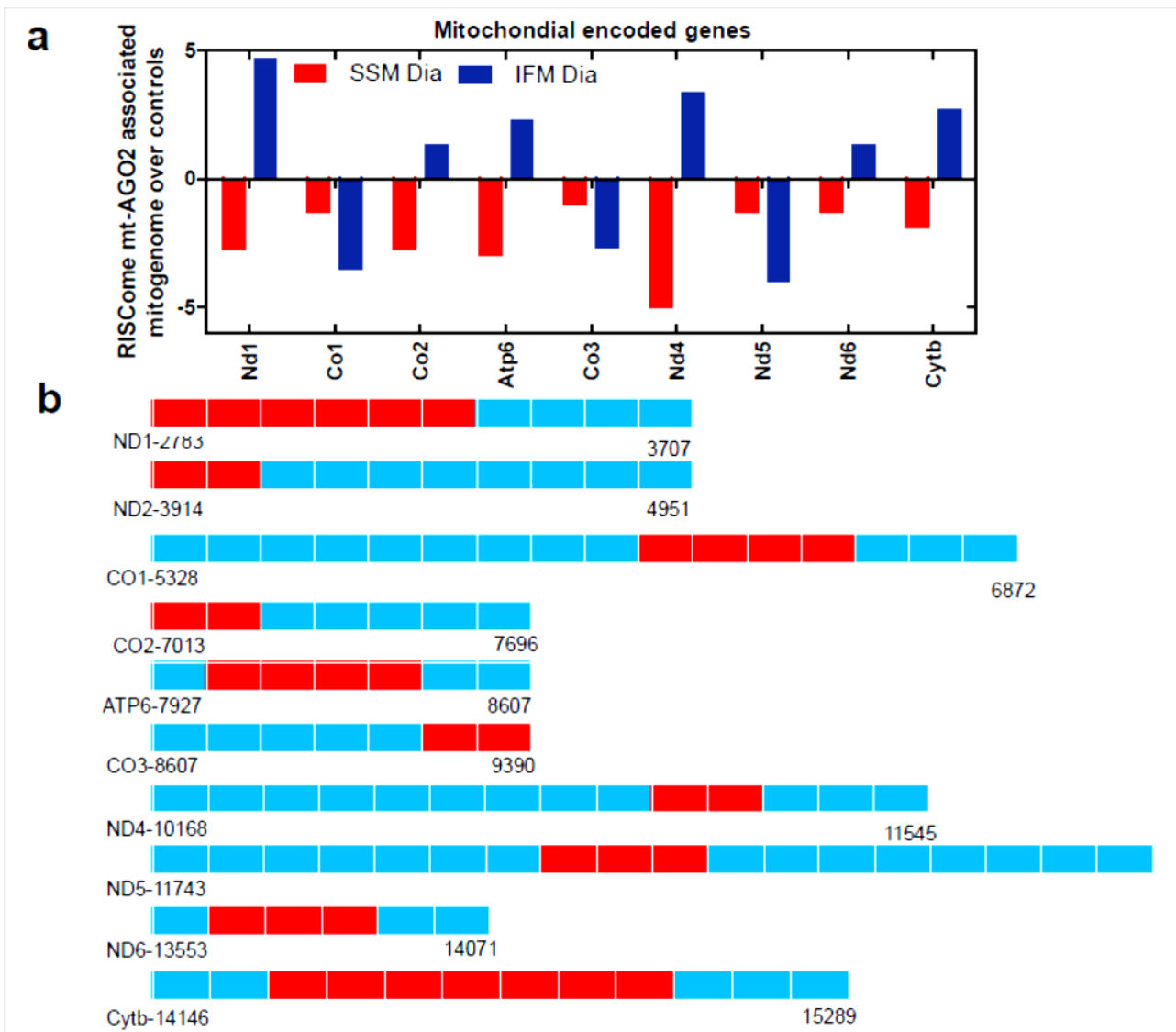


Figure 4.14. Genomic sequencing analyses of mitochondrial RISCome association with mitochondrial mRNA. (a) Mitochondrially-encoded mRNAs in the diabetic subpopulations which were differentially expressed in the mitoRISCome relative to controls. (b) Mapping of the footprint regions of mitochondrial mRNA sequence identified by next generation sequencing which were present in the mitoRISCome. (f) Sequence motif analysis of mitoRISCome associated mitochondrial mRNA, identifying a UUCC rich motif. (g) Distribution of tetra-nucleotide (UUCC) motifs in the mitoRISCome.

Figure 4.15

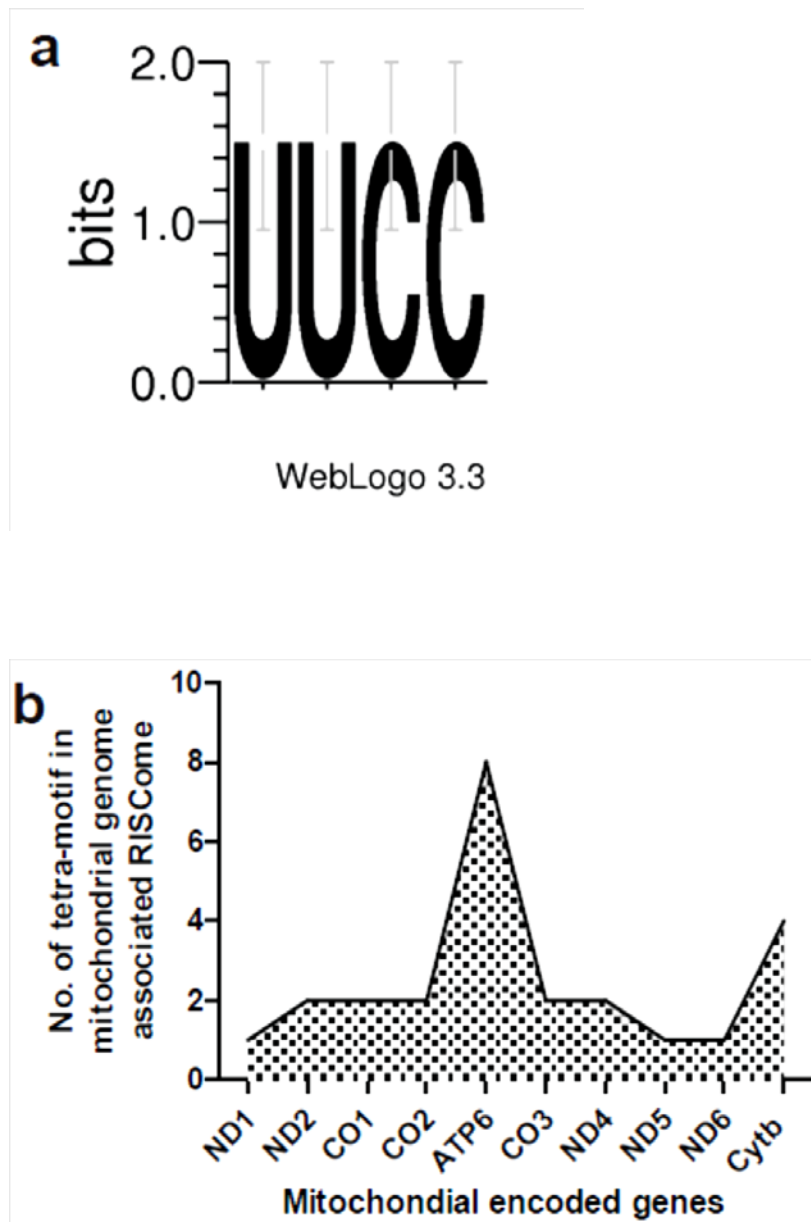


Figure 4.15. Genomic sequencing analyses of mitochondrial RISCome association with mitochondrial mRNA. (a) Sequence motif analysis of mitoRISCome associated mitochondrial mRNA, identifying a UUCC rich motif. (b) Distribution of tetra-nucleotide (UUCC) motifs in the mitoRISCome.

Figure 4.16

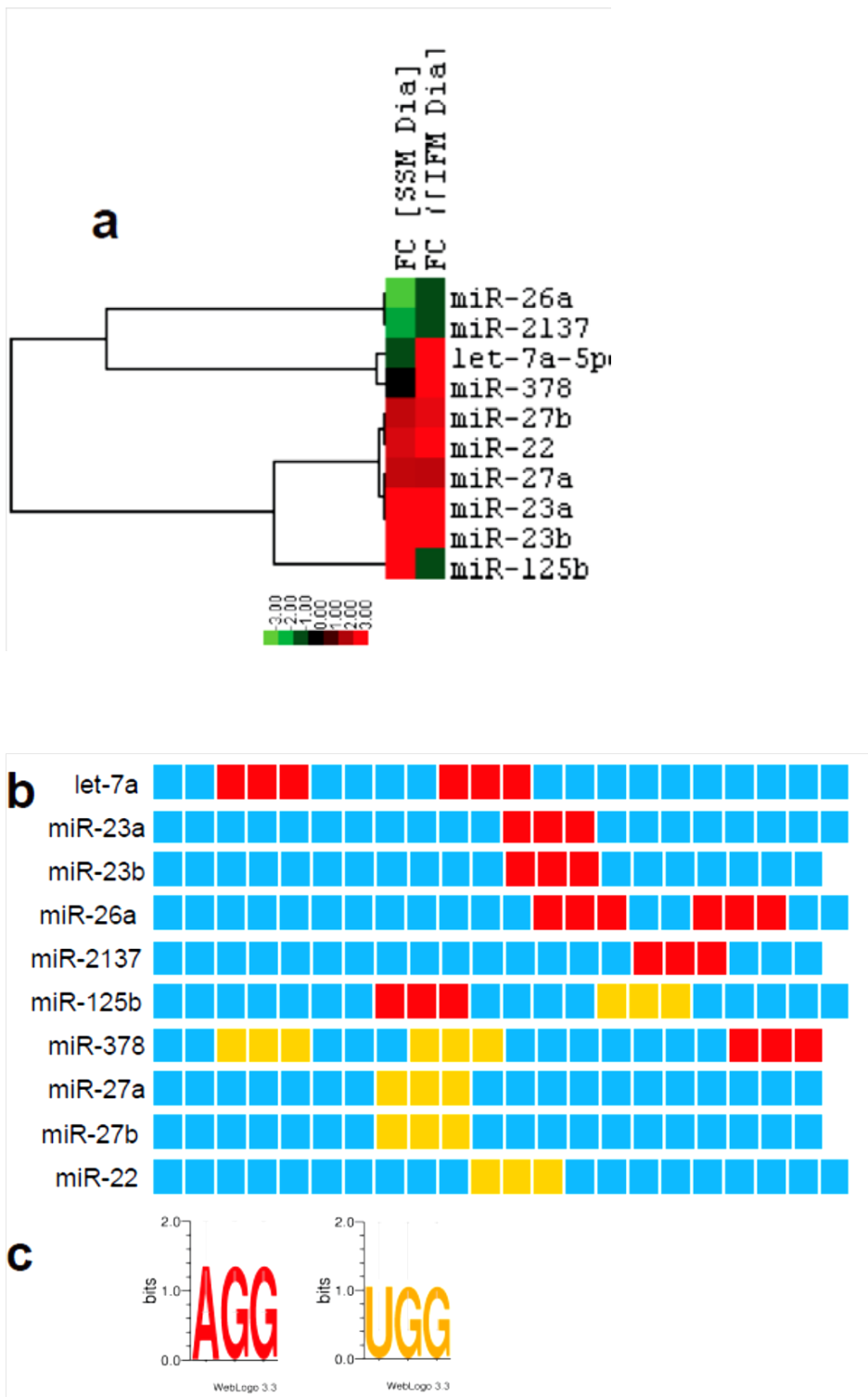


Figure 4.16. Genomic sequencing analyses of mitochondrial RISCome association with mitomiR. (a) MitomiR heat map derived from next generation sequencing of small RNAs identifying enrichment and depletion patterns within the mitoRISCome of diabetic SSM and diabetic IFM, relative to respective controls. (b and c) Mapping of the location of the footprint regions of mitomiR sequence identified by next generation sequencing which were present in the mitoRISCome and were enriched with tri-nucleotide motifs, AGG or UGG. AGG motifs are indicated with red blocks, while UGG motifs are indicated by orange blocks.

Figure 4.17

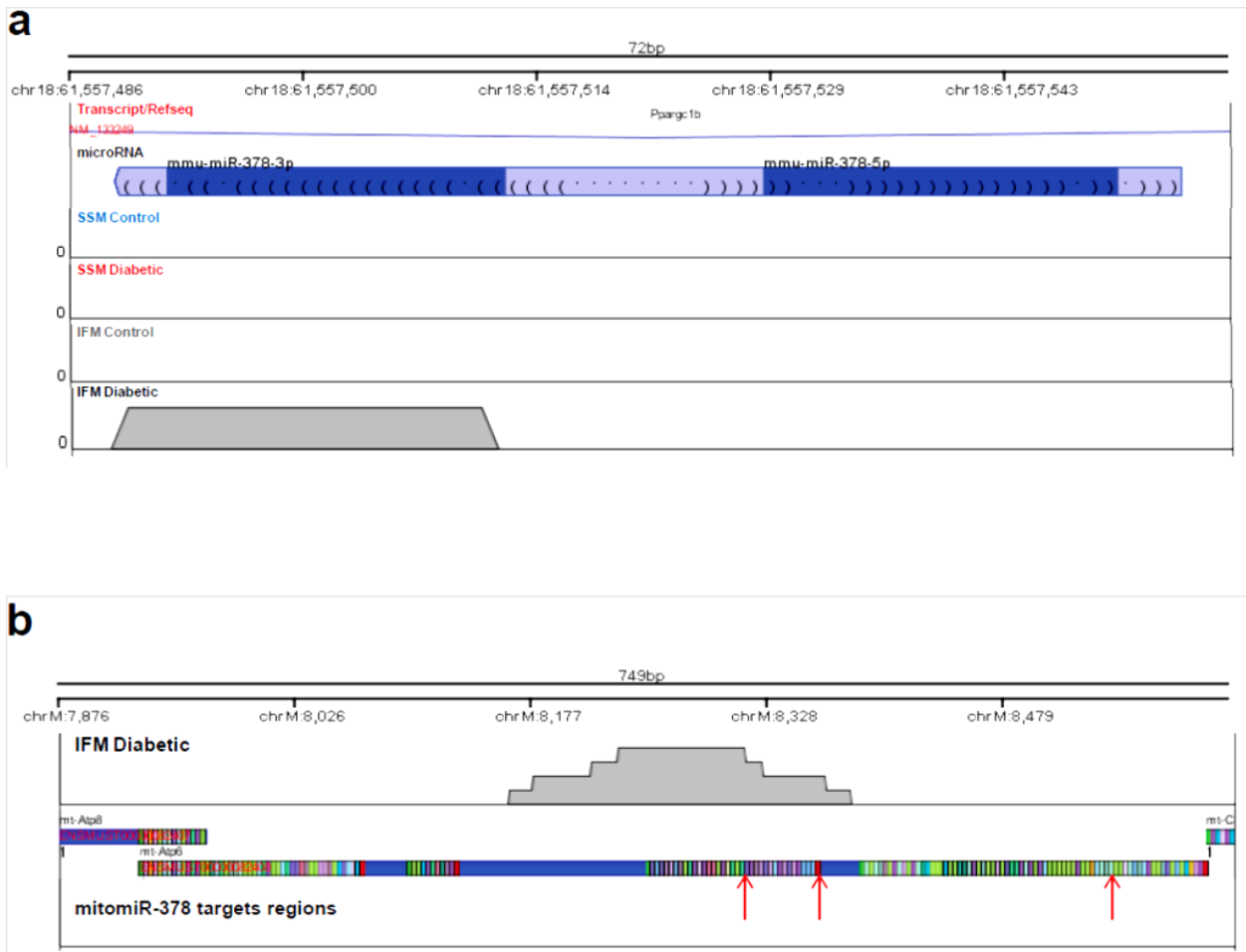


Figure 4.17. Genomic sequencing analyses of mitochondrial RISCome association with mitomiR. (a) Mapping of mitoRISCome associated mitomir-378 enrichment clusters sites in control and diabetic SSM and IFM. (b) MitoRISCome:mitomiR378 ternary maps for the ATP6 target in the diabetic IFM.

Figure 4.18

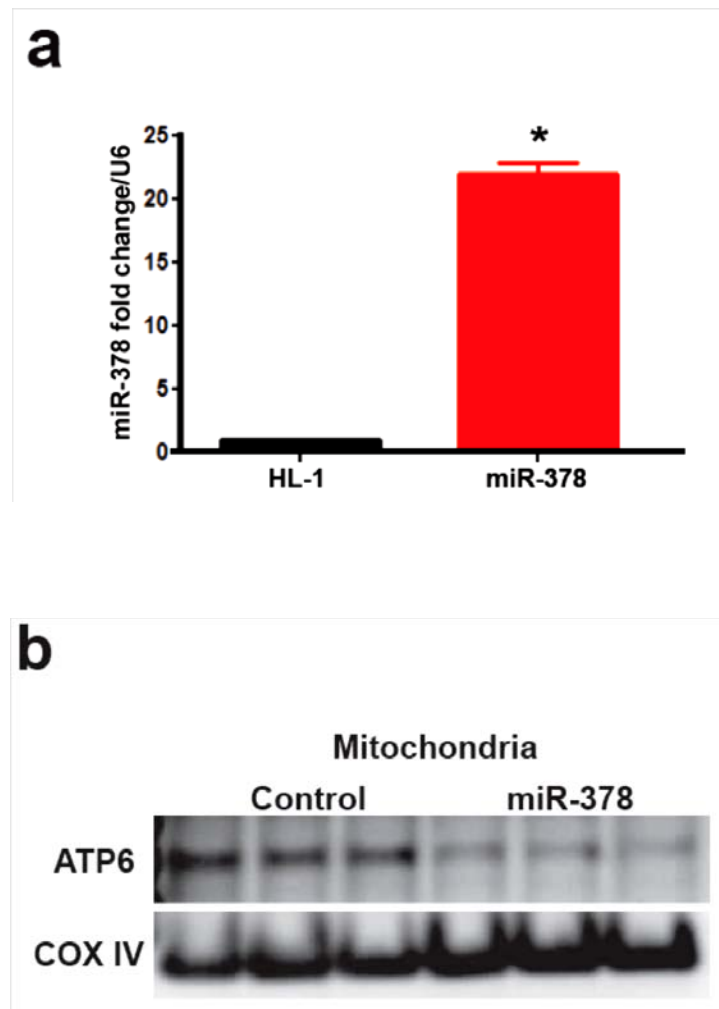


Figure 4.18. Validation of miR-378 mitochondrial targeting. (a) RTPCR analyses for miR-378 levels in isolated mitochondria from HL-1 (Control) and miR-378 cells. (b) Western blot analyses of ATP6 protein levels in isolated mitochondria from control HL-1 cells and in a stable HL-1 cell line expressing miR-378. COX IV protein expression is utilized as a loading control. Values are expressed per COX IV protein levels. Values are means \pm SE, n=6. * $P < 0.05$ for Control vs. miR-378.

Figure 4.19

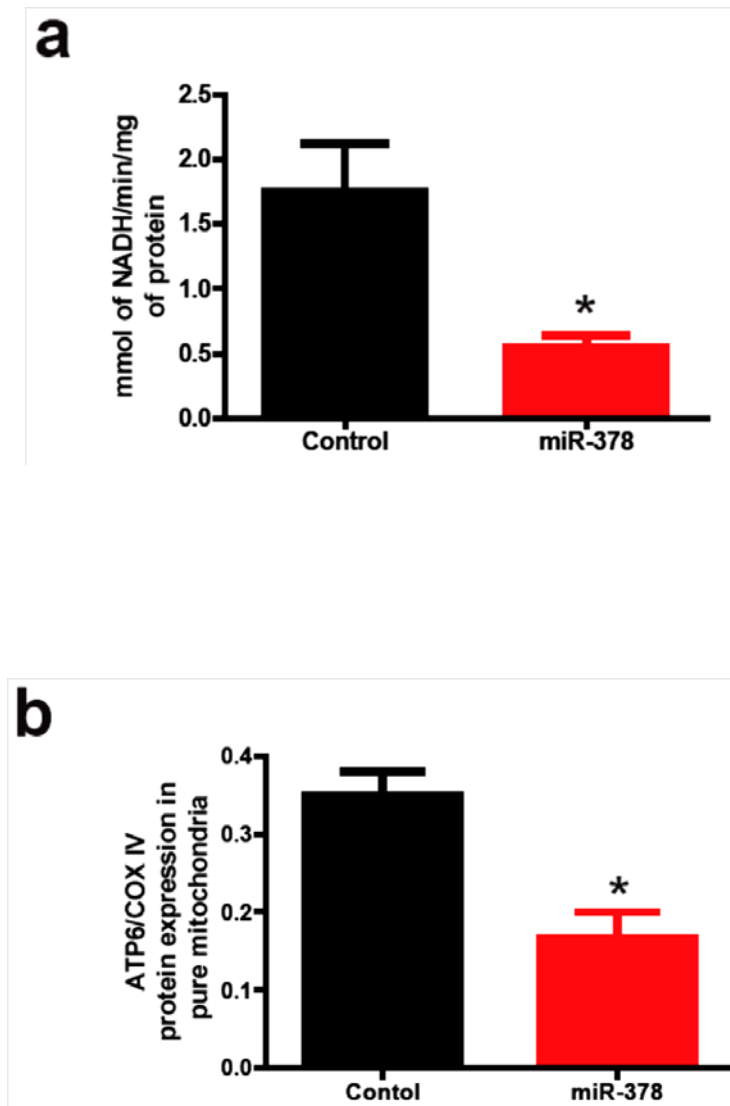


Figure 4.19. Validation of miR-378 mitochondrial targeting and measurement of ATP synthase activity and ATP6 levels. (a) ATP synthase activity in control HL-1 cells and in a stable HL-1 cell line expressing miR-378. Values are means \pm SE, n=6. $*P < 0.05$ for Control vs. miR-378. **(b)** Quantitative analysis of ATP6 protein levels in isolated mitochondria from control HL-1 cells and in a stable HL-1 cell line expressing miR-378. Values are expressed per COX IV protein levels. Values are means \pm SE, n=6. $*P < 0.05$ for Control vs. miR-378.

Figure 4.20

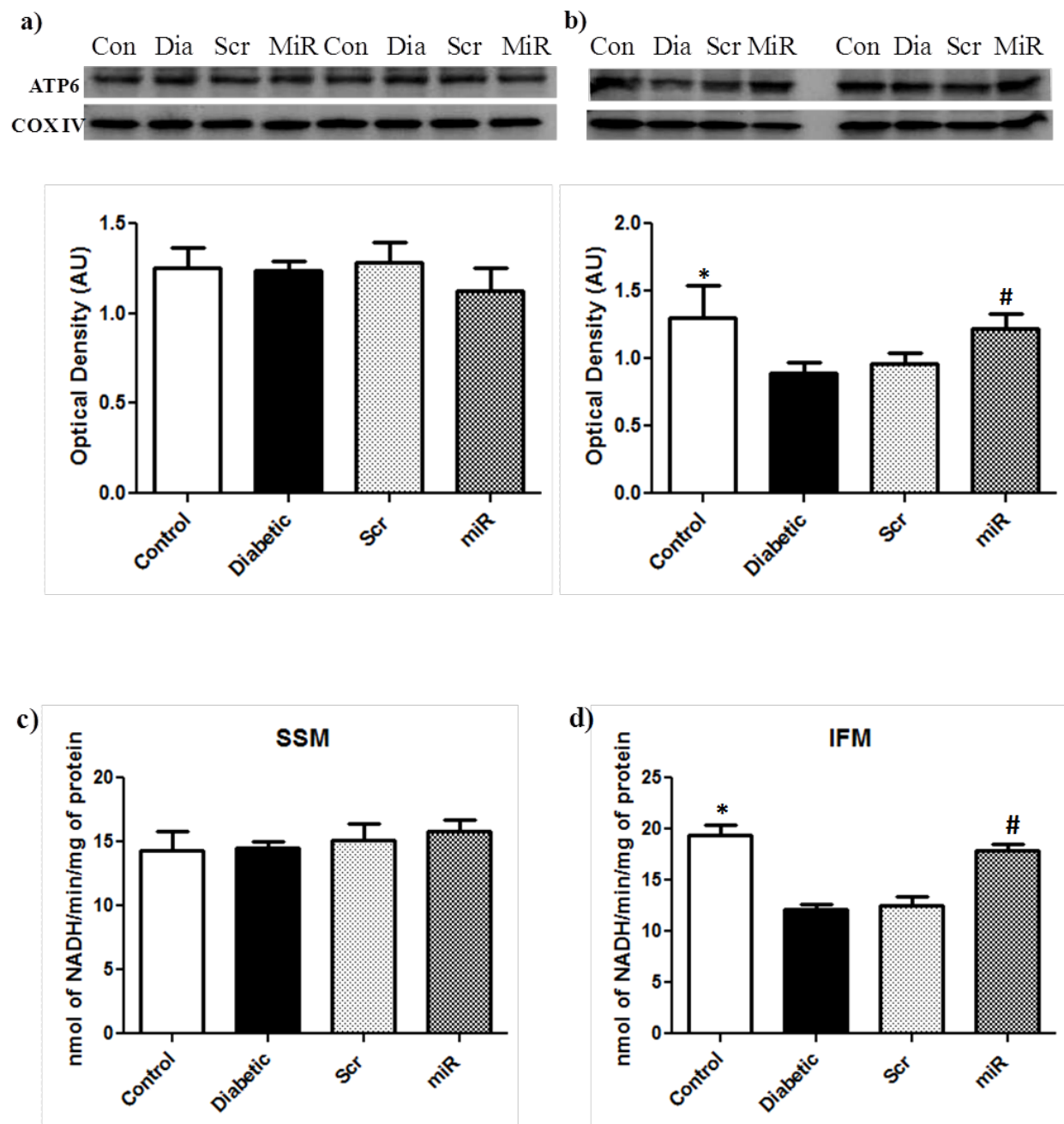


Figure 4.20. ATP6 content and ATP synthase activity with antagomir treatment.

Quantitative analyses by Western blot of ATP6 protein levels following LNA-miR-378 treatment and diabetic induction in **(a)** SSM and **(b)** IFM. COX IV protein expression is utilized as a loading control and values are expressed per COX IV. ATP synthase activities levels following LNA-miR-378 treatment and diabetic induction in **(c)** SSM and **(d)** IFM. Values are expressed as means \pm SE, n=5. * P <0.05 for Control vs. Diabetic and Control vs. Scrambled; # P < 0.05 for LNA-miR-378 vs. Diabetic and LNA-miR-378 vs. Scrambled. ScR = Scrambled; miR = LNA-miR-378.

Figure 4.21.

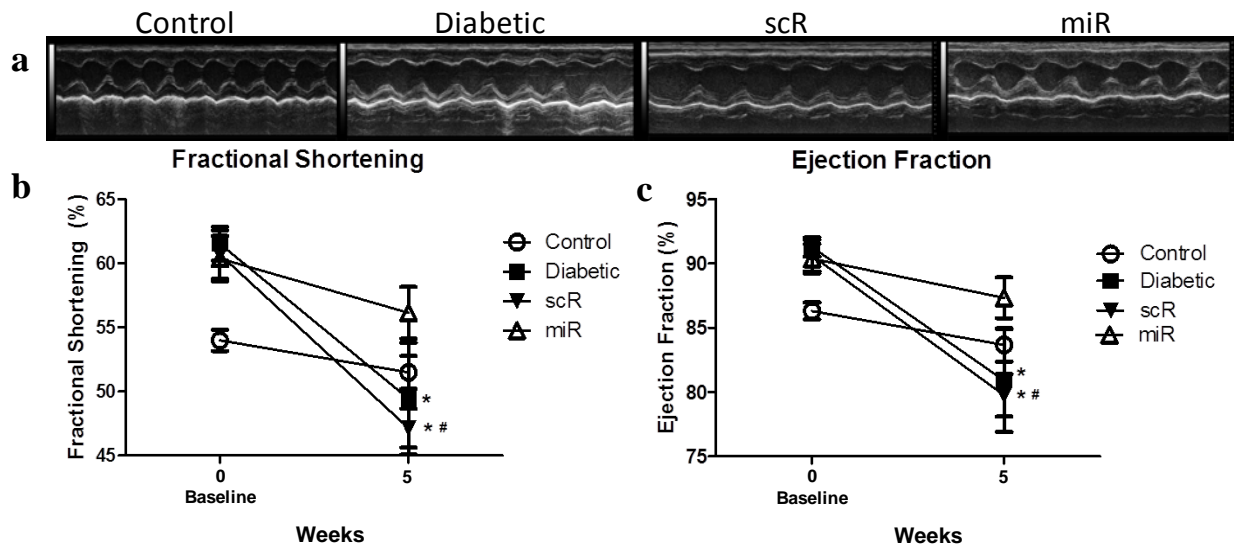


Figure 4.21. Validation of *in vivo* targeting of miR-378 in cardiac contractile function. (a)

Representative M-mode images of Control, Diabetic, Scrambled and LNA-miR-378 treated hearts 5 weeks following diabetes mellitus induction. **(b)** Quantitative summary of % fractional shortening and **(c)** % ejection fraction prior to (baseline) and 5 weeks following diabetes mellitus induction. Values are means \pm SE, n=5. * $P < 0.05$ for baseline vs. 5 weeks; # $P < 0.05$ for LNA-miR-378 vs. Scrambled. ScR = Scrambled; miR = LNA-miR-378.

Figure 4.22

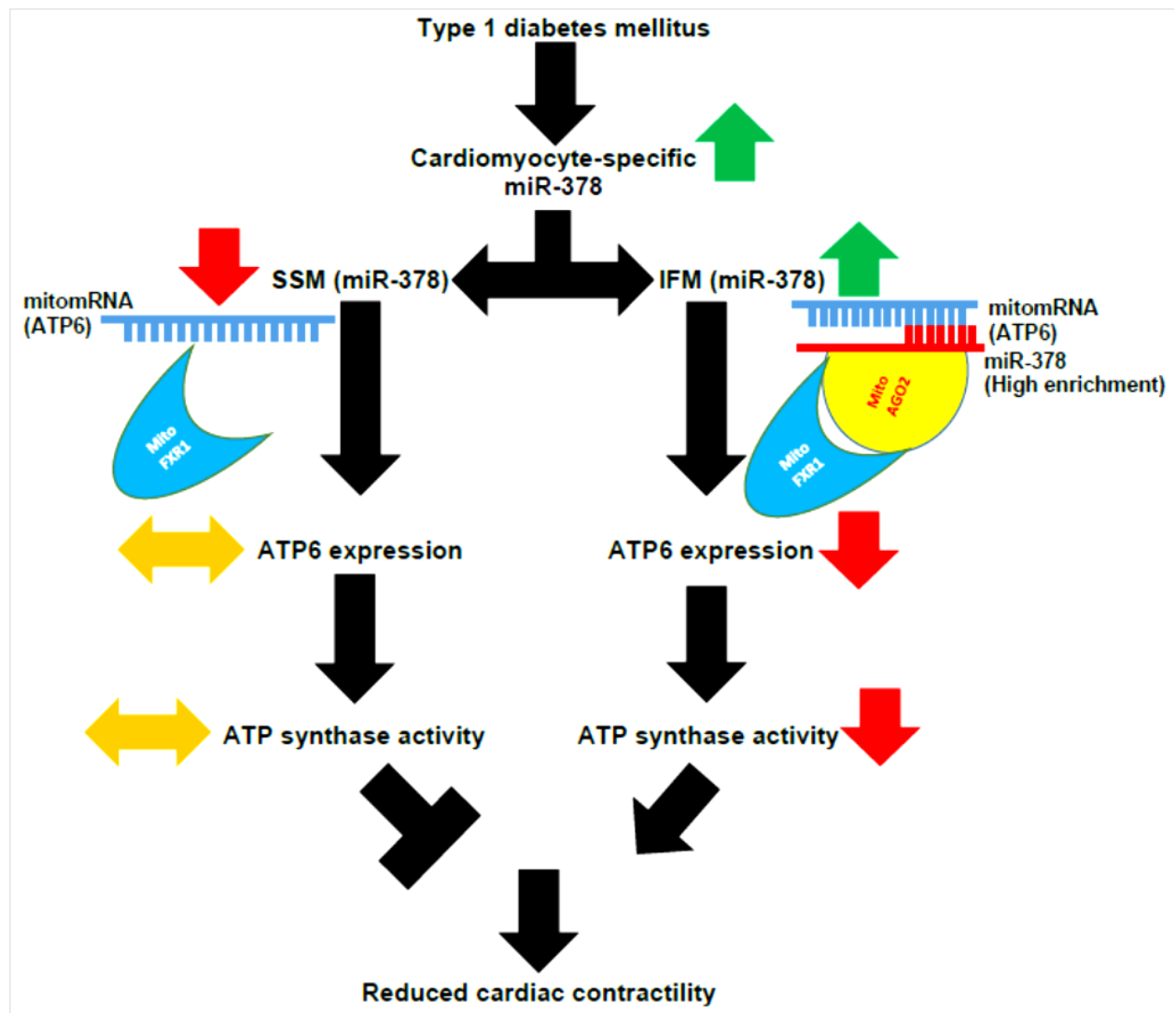


Figure 4.22. Translational regulation of mitochondrial genome-encoded ATP6 protein via miR-378 interaction. Schematic diagram of miR-378 mechanism of action in the translational regulation of mitochondrially-encoded ATP6 and function of the ATP synthase activity during diabetic insult, impacting cardiac contractility.

Chapter 5:

General Discussion

General Discussion

The **overall objective** of this dissertation was to determine the mechanisms involved in maintaining mitochondrial structure as well as examine alterations of miRNA profile in the mitochondrion during type 1 DM. Specifically, we wanted to determine (1) the interaction of mitofilin with MINOS components and investigate novel associations of mitofilin with ATP synthase F0 subunits, (2) the therapeutic benefit of mitofilin overexpression in mitochondrial morphology, dynamics, DNA content, function and ultimately cardiac contractile function and (3) miRNA profile in mitochondrial subpopulations and their role in translational regulation of the mitochondrial genome during type 1 DM. Our **long term goal** is to better understand the mechanisms involved in the pathogenesis of DM for developing therapeutic interventions to protect against diabetic cardiomyopathy. The **central hypothesis** of this dissertation is that alterations in cristae morphology, inner membrane organization and mitochondrial dysfunction during a type 1 diabetic insult are associated with decreased mitofilin content and translational regulation of mitochondrial genome by mitochondrial miRNAs (mitomiRs) respectively. These effects are more pronounced in the diabetic IFM, influencing cardiac contractile processes. Our **rationale** for the proposed research is based on the importance of preserving IMM morphology and mitochondrial genome to attenuate mitochondrial dysfunction. We anticipate being able to determine morphological alterations, mitochondrial genome regulation in mitochondrial subpopulations and investigate areas at risk. The outcomes will provide insight regarding the value of improved mitochondrial structure as well as role of miRNAs in regulating mitochondrial genome in diabetic cardiomyopathy and will further aid in the development of therapeutic interventions that can address mitochondrial subpopulations at risk during type 1 DM.

Cardiovascular complications, including diabetic cardiomyopathy, are the leading cause of morbidity and mortality in type 1 diabetic patients. Mitochondrial dysfunction is central to the pathogenesis of type 1 diabetic heart complications (12, 41, 42, 46). Cardiac mitochondrial analyses are further complicated by the presence of two spatially distinct mitochondrial subpopulations (Figure 1.1). Previously, locales of mitochondrial subpopulations have shown differential effects under a variety of pathological insults including diabetes mellitus (3-5, 12, 24, 28, 29, 36, 54). A review by our laboratory surmises differential impact of various pathologies upon cardiac mitochondrial subpopulations (20). Our laboratory has reported that following a type 1 diabetic insult the IFM exhibit greater dysfunction: enhanced oxidative stress production, decreased size and internal complexity, decreased oxidative phosphorylation, and mitochondrial proteomic alterations (4, 12). Majority of proteins altered during a type 1 diabetic insult are housed within the inner mitochondrial membrane (IMM) and this mitochondrial proteome dysregulation could potentially be associated with the disruption of mitochondrial structure thus affecting crucial mitochondrial functions such as oxidative phosphorylation (4). Among the proteins altered in type 1 diabetic IFM is structural protein, mitofilin, residing in the inner mitochondrial membrane. A list of proteins adapted from proteomic alterations shown by Baseler et al. shows selected proteins from mitochondrial respiratory chain as well as structural protein mitofilin in Table 5.1 (4).

Mitofilin is an inner membrane structural protein established for its role in cristae morphology (23). Various studies have led to the identification of a large inner membrane multi protein complex, critical for inner membrane architecture, mitochondrial functionality, cristae morphology and organization, as well as formation of contact sites and protein biogenesis (1, 7, 19, 21, 47, 51, 57). Mitofilin has been shown to be a critical component of this mitochondrial

contact site and cristae organizing system (MICOS) (38). Interaction of mitofilin with CHCHD3 and Sam50 has been shown to function as mitochondrial intermembrane space bridging complex (MIB) for biogenesis of respiratory complexes (35). Presence of mitofilin in a larger complex with Sam50, metaxins 1 and 2, CHCHD3, CHCHD6 and DnaJC11 has been shown to potentially regulate protein import (56). Hence, interactions of mitofilin with mitochondrial inner and outer membrane proteins and their roles in proper functioning of mitochondria have been studied.

Protein Name	SSM Diabetic/ SSM Control	IFM Diabetic/ IFM Control
Mitochondrial Respiratory Chain		
ATP synthase, H ⁺ transporting, FO complex subunit F	1.27	.61
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	NS	.88
NADH dehydrogenase ubiquinone flavoprotein 1	NS	.83
NADH dehydrogenase ubiquinone 1 alpha subcomplex 8	1.21	NS
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 12	NS	.60
Cytochrome C oxidase subunit VIb polypeptide 1	NS	.68
Structural Protein Inner membrane protein Immt, Mitofilin	NS	.64

Table 5.1. Proteomic alterations in type 1 diabetic IFM (4).

With various roles of mitofilin in different protein complexes being reported, we examined potential association of mitofilin in mitochondrial subpopulations in Chapter 2. Further, mitochondrial subpopulations specific interactions were studied during type 1 diabetes mellitus. No study has examined mitochondrial subpopulations specific interaction of mitofilin. We for the first time report novel association of mitofilin with ATP5F1 and present our findings of mitofilin interaction with CHCHD3 in mitochondrial subpopulations during type 1 diabetic insult. Proteins pulled down with mitofilin constituted proteins from ETC complexes I-V, protein import machinery and mitochondrial structural proteins. Potential roles of mitofilin in regulating respiratory complexes (35), protein import (56) and mitochondrial structure (21, 23, 57) are in

agreement to our findings. Further, presence of C-terminal domain of mitofilin has been reported as a pre-requisite for interaction of mitofilin with Tob55/Sam50 complex that could potentially regulate outer membrane protein import and formation of crista junctions (26).

CHCHD3 and mitofilin have been shown to regulate cristae morphology, mitochondrial function and are constituents of the MICOS complex (13, 21, 38). Previous studies from our laboratory and others have shown decreases in mitochondrial size, internal complexity and structural alterations specifically in the IFM subpopulations (12, 42). Further, proteomic studies from our laboratory show decreased mitofilin content in T1DM IFM subpopulations (4). These findings led us to examine CHCHD3 content and interaction with mitofilin during diabetic insult. Assessment of interaction between mitofilin and CHCHD3 in control and diabetic mitochondrial subpopulations revealed decreased association of mitofilin with CHCHD3 in diabetic IFM which could potentially lead to morphological alterations observed during diabetes. Further, protein content of CHCHD3 was not altered in either of the subpopulations during diabetes, strengthening the role of mitofilin in cristae morphology and function. With that being said, assessment of mitofilin interaction with other constituents of the MICOS complex in mitochondrial subpopulations during diabetes mellitus could provide insight into mechanisms regulating mitochondrial structure and functions. Further, studies examining MICOS content in control and diabetic mitochondrial subpopulations could provide subpopulation specific role of the complex in generation of cristae morphology and inner membrane architecture.

Other mitochondrial proteins, not reported to be in a multi protein complex with mitofilin, have been implicated in regulation of cristae morphology (16, 31, 37, 39). A study by Piquereau et al. examining down-regulation of optic atrophy 1 (OPA1) in mouse model reported presence of clusters of fused mitochondrial network and altered cristae (39). Study of another

structural protein prohibitin present as assembly of two homologous prohibitin subunits, prohibitin1 and prohibitin2 have been shown to control cristae morphogenesis and potentially led to functional compartmentalization in the inner membrane of the mitochondria (31). Of particular interest to our study in Chapter 2 was findings reported by Paumard et al. who utilized yeast cells to examine potential role of ATP synthase subunits e or g. These authors present potential role of these subunits in regulating cristae morphology (37). Further, another study by Rabl et al. showed that deletions of either of these subunits led to reduction in cristae tip numbers and crista junctions diameter enlargement. These authors showed genetic interaction of mitofilin with ATP synthase subunits e or g (40). Proteins pulled down with mitofilin, presented in Chapter 2 did identify proteins of the ATP synthase subunits. These findings of cristae regulation by ATP synthase subunits combined with subunits pulled with mitofilin identified in our study led us to assess novel subpopulations specific association of mitofilin in control and diabetic groups.

Novel association of mitofilin with ATP synthase subunit b (ATP5F1) was observed in both mitochondrial subpopulations. Association of IFM diabetic was decreased when compared to the control. Previous findings from our laboratory have shown decreased ATP synthase activity in diabetic IFM (3, 12). Decreased association of mitofilin with ATP5F1 in diabetic IFM could potentially alter ATP synthase functionality. Examination of other ATP synthase F0 subunits pulled down with mitofilin would further aid in understanding subpopulation based association of mitofilin. Upon examination of mitofilin association comparing control SSM and IFM subpopulations, increased association is observed in IFM, suggesting a possible mechanism that could result in increased ATP synthase activity in the IFM. Hence, novel association of mitofilin with ATP5F1 could present a different mechanistic phenomenon in regulation or cristae

morphology. Differences of mitofilin association present in diabetic IFM could result in structural as well as functional decrements observed in diabetic IFM subpopulations during a type 1 diabetic insult.

Because of decreased association of mitofilin with ATP5F1 in diabetic IFM and a lot of proteins of Complex V subunits being pulled with mitofilin, we sought to identify whether this decreased association resulted in any alteration of the total complex content in mitochondrial subpopulations during diabetes. We utilized BN-PAGE to examine complex V content of SSM and IFM in both control and diabetic groups. No significant differences were observed in ATP synthase content in SSM and IFM. These findings indicate that decreased association observed between mitofilin and ATP5F1 is not sufficient to change the overall protein content. Further, examination of other ETC complexes in control and diabetic mitochondrial subpopulations have shown no changes in protein content of any complexes (45).

It should be noted that all the proteins pulled down with mitofilin do not directly interact with it. ATP synthase F1 subunits proteins, residing in the matrix of the mitochondrion were also pulled with mitofilin. Association of ATP synthase F0 subunits with F1 subunits could result in the pull down of these proteins thus suggesting no direct association of mitofilin with them. Indeed, when association of mitofilin with ATP synthase F1 subunit alpha (ATP5A) was assessed, no association was observed (data not provided). Hence, careful assessment and analyses should be performed in order to examine novel association of mitofilin. One way of designing experiment would be to consider location of the protein and utilization of immunoprecipitation using both mitofilin and protein of interest vice versa. Also, assessment using BN-PAGE would allow examining protein complexes. A clear understanding of protein

complexes formed by mitofilin and its potential role in mitochondrial functions greatly aids in investigating these novel associations.

Taken together, the data compiled in Chapter 2 indicates that examination of novel and known mitochondrial subpopulations specific association of mitofilin could potentially regulate mitochondrial structure and function during a type 1 diabetic insult. Further, key processes involving ETC complexes, protein import as well as mitochondrial structure could be affected and potentially dependent upon the association partners of mitofilin.

Decrements of mitofilin have been observed in many human diseases such as Down's syndrome (6, 33), Parkinson's disease (48, 49), Epilepsy (17, 34), Neurodegeneration (52, 55) and type 1 diabetes mellitus (4, 9). Studies implementing down-regulation of mitofilin in HeLa cells have shown abnormal mitochondrial morphology with concentric layers of inner membrane. These authors have reported reduced cell proliferation, increased apoptosis and elevated reactive oxygen species (ROS) production (23). Similar depletion studies in yeast cells and *C. elegans* have resulted in curved and stacked cristae tubules, increased ROS production and reduction in cristae junctions which is associated with decreased DNA content (32, 40). In the type 1 diabetic heart, the focus of this study, abnormal mitochondrial morphology with decreased cristae density has been reported (9). Studies examining over expression of mitofilin in the context of diabetes have not been implemented. Over expression of mitofilin in yeast cells resulted in increased diameter as well as branching of cristae and cristae junctions (40). We next sought to identify potential effects of mitofilin overexpression in mitochondrial structure, function, DNA content, ROS generation and cardiac function, the findings of which are presented in Chapter 3 of this dissertation.

In order to examine the role on mitofilin in a STZ induced type 1 diabetes model (Chapter 3), we generated a transgenic mouse model overexpressing mitofilin which constituted a significantly higher protein expression in IFM as compared to SSM. These findings are in agreement with a study by Ferreira et al. who showed a 37 fold higher mitofilin content in cardiac IFM than that in SSM subpopulations (15). With mitofilin downregulation shown to alter mitochondrial morphology, we utilized electron microscopy to examine structural changes in diabetic mitochondria and further analyzed the effects of mitofilin overexpression in our diabetic models. Damaged mitochondrial structure was observed in diabetic IFM when compared to control. These findings are in agreement to studies by John et al. who observed similar morphological alterations with mitofilin down regulation (23). Our transgenic mouse model overexpressing mitofilin displayed preservation of mitochondrial morphology and cristae structure in the face of diabetes mellitus which corroborate the findings by Rabl et al. who showed increased cristae junction formation and branching with mitofilin overexpression in yeast cells (40). Decrements in mitochondrial internal complexity and granularity observed in type 1 diabetic IFM was also preserved with mitofilin overexpression. Taken together these findings further strengthen the role of mitofilin in maintaining cristae morphology and hence overall mitochondrial structure.

The inner mitochondrial cristae membrane is the principal site for ATP production through oxidative phosphorylation (OXPHOS) (18) and improved cristae morphology increases the inner membrane surface area thus enabling a greater capacity for OXPHOS and ATP production. We next examined ETC complexes and ATP synthase function in our transgenic mouse model. Decrements in ETC complexes I, III, IV, and V observed in type 1 diabetic IFM was restored with mitofilin overexpression. This restoration in ETC complexes and ATP

synthase function could be associated with better mitochondrial structure as well as internal complexity observed in our transgenic animals. Though not examined in this dissertation, increased inner membrane surface area as a result of improved mitochondrial structure in our transgenic diabetic animal models could potentially lead to an enhanced capacity for OXPHOS and ATP generation.

One potential mechanism that could increase OXPHOS and ATP generation could be due to changes in absolute contents of ETC complexes. In order to examine total ETC protein content we utilized a BN-PAGE approach. As presented in Chapter 3, we did not observe any changes in the absolute contents of ETC complexes. These findings suggest that mitochondrial functional benefits observed within our transgenic mouse model are not the result of increased ETC complexes but due to preservation of IMM integrity that could enhance stabilization of the ETC complexes. Role of mitofilin in maintaining IMM integrity and architecture has been reported (19, 21). However, future studies aimed at examining cristae surface area and their impact upon IMM architecture, ETC stabilization in the presence of mitofilin overexpression would aid in understanding key mechanisms contributing towards observed OXPHOS changes.

In an effort to better understand the role of mitofilin overexpression, we next sought to examine mitochondrial dynamics and assess if it affects fission and fusion processes. Examination of proteins involved in regulation of mitochondrial fission and fusion showed no differences in SSM and IFM. Western blot analyses of the proteins examined (mitofusin 1 and 2, OPA1, Dynamin- related protein 1) showed no alterations in diabetes and with overexpression of mitofilin. These findings are in agreement to a study by John et al. who reported normal mitochondrial fission and fusion with mitofilin down regulation (23). These findings suggest that no alterations in mitochondrial size and number were observed with overexpression of mitofilin.

Further, assessment of mtDNA content in control vs. transgenic mitofilin animals showed similar copy numbers.

Enhanced ROS production has been reported from cardiac mitochondria of different type 1 diabetic models (12, 43). The mitochondrial respiratory chain is the major source of ROS generation and ETC complexes I and III are culpable of electrons leakage thereby generating incompletely reduced forms of oxygen (8, 44). The oxidative milieu resulting from mitochondrial ROS can cause cellular damage by oxidation of proteins, conversion of lipids to form lipid peroxidation products. Increased oxidative damage as shown by nitrotyrosine residues and lipid peroxidation has been observed in type 1 diabetic IFM (12). Because of improvement in mitochondrial cristae structure that houses the complex contributing in ROS generation, we examined lipid peroxidation with mitofilin overexpression in control and diabetic animals. Our findings as highlighted in Chapter 3 show attenuation of lipid peroxidation in diabetic IFM which could potentially be due to preservation of ETC function and mitochondrial structure. Further, improved cristae structure could potentially attenuate electron leakage resulting in decreased ROS production and downstream oxidative damage.

Significant decrements in ejection fraction, fractional shortening, and cardiac output have been reported in type 1 diabetic heart (3). Restoration of mitochondrial structure and function by mitofilin overexpression led us to examine if cardiac contractile function was affected. Mitofilin overexpression led to the preservation of ejection fraction and fractional shortening, suggesting improved cardiac contractile function which could be the result of better mitochondrial structure and ultimately, function.

Taken together, the results garnered from chapter 3 highlight the novel ability and role of mitofilin in restoration of mitochondrial structure, function, and ultimately cardiac contractile function during type 1 diabetic insult. Well established role of mitofilin in maintaining cristae morphology, IMM architecture and integrity, ETC stabilization, and protein biogenesis (1, 7, 19, 21, 40, 47, 51, 57) supports our findings in Chapter 3 of this dissertation.

The structure of IMM is crucial for proper mitochondrial functioning as it houses the ETC and OXPHOS machinery. Role of mitofilin has been reported for proper maintenance of IMM architecture, integrity and stability through its role in cristae morphology. However, decrements in cristae junctions, altered cristae morphology, decreased mitochondrial size and internal complexity and reduced ETC and OXPHOS activities have been reported in diabetic IFM (9, 12). Moreover, proteomic alterations specific to the diabetic IFM subpopulations reveal decrease in structural as well as proteins of the ETC and OXPHOS complexes (4). Altered cristae morphology in conjunction with decrements in proteins of ETC complexes could potentially lead to damage in IMM architecture and thus increased ROS generation as observed in type 1 diabetic IFM (12). In order to address proteomic alterations in diabetic IFM, we have examined protein import in the mitochondrion and reported a decrease in import of nuclear encoded proteins in the diabetic IFM (4). In addition to this, utilization of a mitochondria phospholipid hydroperoxide glutathione peroxidase 4 (mPHGPX) overexpression mouse model, revealed a reversal of proteomic alterations, dysfunctional protein import and ultimately mitochondrial function. Further, posttranslational modifications including oxidations and deamidations were attenuated in diabetic IFM with mPHGPX overexpression (3). Potential regulation of the nuclear encoded proteins with miRNAs has also been examined (5). However, these were more geared towards the nuclear encoded proteins.

The mitochondrial genome encodes for 13 proteins constituting ETC complexes I, III, IV, and V. Examination of translational regulation of these proteins is crucial to comprehend mitochondrial dysfunction observed in type 1 diabetes. Further, development and progression of many human diseases involves regulation of mitochondrial genome (27, 30). In order to address potential regulation of the mitochondrial genome during type 1 diabetic insult, we examined alterations of miRNAs profile in mitochondrial subpopulation and further assessed its potential roles in regulating the mitochondrial genome. The findings of our study are presented in Chapter 4 of this dissertation.

Although presence of miRNAs in the nucleus and processing bodies have been reported (22), identification in the mitochondrion are very limited (14). Using prediction analyses 46 mitochondrial miRNAs (mitomiRs) were predicted to bind to mitochondrial genome-encoded mRNAs with over half of the mitomiRs potentially regulating multiple mitochondrial genome-encoded mRNAs. Further, microarray analyses of mitomiRs in cardiac mitochondrial subpopulations revealed dramatically altered and reciprocal patterns. Of the identified mitomiRs, mitomiR-378 displayed a reciprocal response in cardiac mitochondrial subpopulations with over 20 fold increase in diabetic IFM. Examination and assessment of potential roles of mitomiR-378 during type 1 diabetic insult were performed in our studies. Dramatic increase of mitomiR-378 only in the IFM might be of particular relevance specifically in the IFM and contribute in its functionality. Indeed, ATP6, a mitochondrial genome target mRNA for mitomiR-378 is decreased in conjunction with ATP synthase activity thus suggesting a regulatory role for mitomiR-378 in type 1 diabetic IFM.

In an effort to better understand the regulatory role of mitomiR-378, we conducted both *in vitro* and *in vivo* experiments. Overexpression of miR-378 in a cardiac HL-1 cell line resulted

in decreased ATP6 protein content and ATP synthase activity. Assessment of miR-378 levels in mitochondria isolated from over expressed cell lines revealed a 22 fold increase in miR-378 levels, confirming regulatory role of the miRNA. Multiple intra-peritoneal injections of an LNA-linked antagomiR targeting miR-378 (LNA-miR-378) were utilized to assess the *in vivo* efficacy of miR-378. Indeed, down-regulation of ATP6 protein content, ATP synthase activity, and improved cardiac pump function were observed with LNA-miR-378. These findings suggest the potential role of enhanced mitomiR-378 for regulating mitochondrial genome-encoded ATP6 content and contributing to bioenergetics deficit observed during type 1 diabetes mellitus. Regulatory role of miR-378 in cardiac remodeling and cell survival have been reported by other studies as well (25).

Post-transcriptional gene regulation by miRNA requires binding to complementary sites on the target mRNA. Further, association of bound miRNA and mRNA with RNA-induced silencing complex (RISC) is crucial for translational regulation of mRNA (2). Recruitment of RISC components, including argonaute-2 (Ago2) and fragile X mental retardation-related protein 1 (FXR1) has been shown to influence translation (50). We next sought to examine and identify the presence of RISC components in the mitochondrion. Western blot analyses revealed differential expression of Ago2 and FXR1 in the mitochondrion relative to control with the response being subpopulation-specific in nature. Significant decrease in Ago2 was observed in both subpopulations with diabetic insult whereas FXR1 was decreased in SSM but increased in IFM subpopulations. These findings confirm the presence of RISC components in the mitochondrion and suggest the potential of RISC formation in the mitochondrion.

Cross linked immunoprecipitation (CLIP) experimental approach have been previously utilized to identify direct miRNA/mRNA/protein interactions to confirm direct association

between nucleotide and protein components (11). Further, direct interaction of these molecules is an absolute requirement to facilitate a functional cytoplasmic regulatory complex (10, 11, 53). Using similar experimental technique coupled with next generation sequencing we determined binding maps and sequence motifs of miRNA-378 binding directly to the coding region of ATP6 mRNA. Our findings indicate that mitomiR-378 targets ATP6 coding region to control its translational activity. Further, translational regulation of ATP6 required the presence of both Ago2 and FXR1 presence in the RISC as observed in diabetic IFM. Absence of mitomiR-378 or limiting content of Ago2 as observed in diabetic SSM doesn't result in translational regulation.

In summary, chapter 4 of this dissertation reports translational regulation of mitochondrially-encoded proteins by mitomiRs during type 1 diabetic insult. This regulatory mechanism requires the presence of a specific mitochondrial RISC component. These findings bring forth an interesting mechanism that can be utilized to study translational regulation of the mitochondrial-encoded genome in different pathological insult.

Findings from these studies clearly implicate mitochondrial structural abnormalities as a crucial contributor to mitochondrial dysfunction observed during type 1 diabetic insult. Down-regulation of mitofilin protein content in type 1 diabetic IFM not only resulted in its decreased association with previously reported and novel proteins (Chapter 2), it resulted in altered cristae morphology, and decreased internal complexity of the mitochondrion (Chapter 3). Further, overexpression of mitofilin maintained cristae structure and functions of mitochondria leading to preservation of cardiac contractile function (Chapter 3). Observed proteomic and functional dysregulation in ETC complexes of diabetic IFM led us to examine the translational regulation of mitochondrial-encoded proteins by mitomiRs (Chapter 4). Mitochondrially-encoded proteins constitute ETC complexes I, III, IV, and V. Indeed, antagomir treatment targeted at miR-378 led

to preservation of ATP6 protein, ATP synthase activity, and cardiac pump function during type 1 diabetic insult (Chapter 4).

The overall summary of this dissertation examines influences of mitochondrial structural abnormalities as well as role of mitochondrial miRNAs in regulation of mitochondrial genome and hence functions. Examination of ETC and OXPHOS defects in cardiac mitochondria during T1DM were assessed by examining two different aspects (Figure 5.1). Down-regulation of mitofilin in diabetic IFM results towards a decreased trend in association of mitofilin with CHCHD3 that forms the MICOS complex which has been shown to regulate IMM architecture, integrity and mitochondrial cristae morphology. Decreased association of mitofilin could result in damaged mitochondrial morphology, decreased internal capacity as well as decreased in ETC and OXPHOS activities that are housed in the cristae of the IMM. Decreased ETC and OXPHOS machinery results in increased ROS production as observed in type 1 diabetic IFM. Increased oxidative milieu causes posttranslational modifications including oxidations and deamidations. Increased ROS generation from the ETC complexes was attenuated by mitofilin overexpression with preservation of mitochondrial structure and function. One other potential mechanism resulting in decreased ETC and OXPHOS machinery could be due to decrements in protein constituents of the ETC complexes. Nuclear encoded proteins and their regulation by miRNAs have been previously reported. However, translational regulation of mitochondrially-encoded proteins that constitute ETC and OXPHOS machinery by miRNAs residing in the mitochondrion has not been studied. Examination of this facet of the mitochondrion led to examination of miRNAs in the mitochondrion termed as mitomiRs. Indeed, dysregulation of mitomiRs in a reciprocal pattern were observed in cardiac SSM and IFM subpopulations. Further, both *in vitro* and *in vivo* experiments examining miR-378 led to preservation of ATP6 protein and

functionality. Studies directed at restoring mitochondrial structure as well as targeted at mitomiR-378 regulation led to restoration of mitochondrial function and ultimately cardiac contractile function.

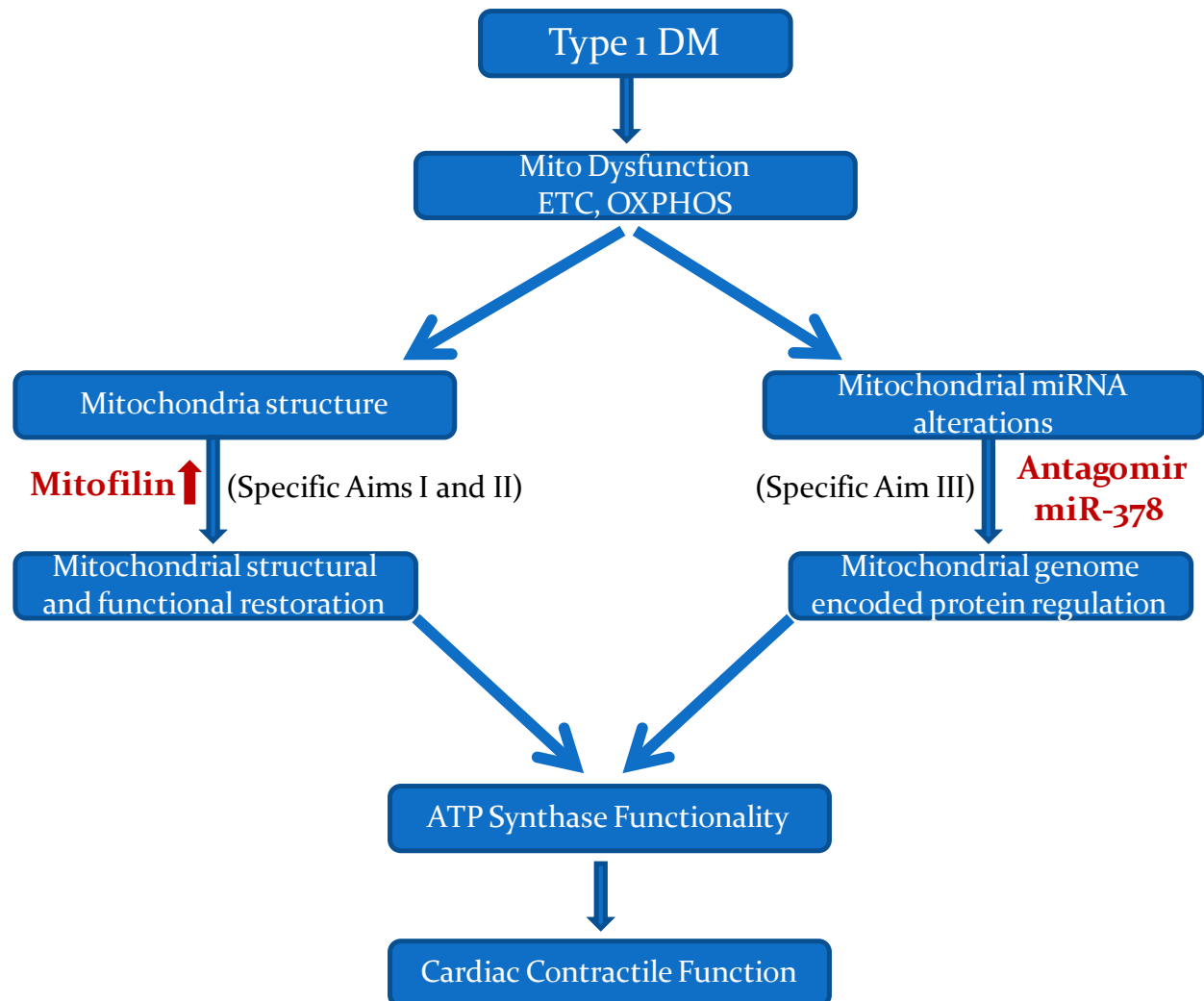


Figure 5.1: Schematic representation of mitochondrial dysfunction assessment through examination of mitochondrial structure and mitochondrial miRNAs.

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Chapter 6:

Future Directions

FUTURE DIRECTIONS

The findings of this dissertation highlight the importance of mitochondrial structure, morphology, architecture and role of IMM for proper mitochondrial functioning. Specifically, as it relates to mitochondrial structure, the role of mitofilin, a mitochondrial structural protein located at the IMM has been well studied and examined for its role in mitochondrial structure and functioning during T1DM. Moreover, overexpression of mitofilin, through a novel transgenic mouse model created in our laboratory, led to restoration of mitochondrial structure, function and cardiac function during T1DM. Also this dissertation has well established the role of miRNAs in regulating mitochondrial genome encoded proteins and their functions. Furthermore, utilization of antagomir delivery to regulate miR-378 led to restoration of mitochondrial function and ultimately cardiac pump function during T1DM. Taken together these findings warrant the assessment of mitofilin as a therapeutic intervention for patients with T1DM. This dissertation not only identifies mitofilin as a novel therapeutic target, but also presents ways that can be utilized to regulate mitofilin protein levels through miRNAs by antagomir treatment. Future studies centered at regulating mitofilin through antagomir delivery approach provide a novel platform for T1DM therapeutics.

One way to regulate mitofilin levels in the T1DM cardiac mitochondria would be to deliver antagomir that are directed towards binding with miRNAs that regulate mitofilin. In doing so, potential downregulation of mitofilin content by miRNAs would be attenuated by inhibition through antagomirs. miRNAs are 22 nucleotides small non-coding evolutionarily conserved RNAs that regulate mRNAs (4, 5, 7). miRNAs regulate mRNA function by binding to an 7 base pair complementary seeding region and can either degrade mRNAs or inhibit their translation (10, 11). Antagomirs are anti-sense oligonucleotide sequences conjugated with

cholesterol that can be systemically delivered by injection. They have a high binding affinity to RNA and DNA and have sustained miRNA blockade (13, 15). Further, utilization of antagomirs for miRNA blockade is currently under phase II clinical trials for a number of different pathologies (14, 24). Hence, utilizing antagomirs to inhibit miRNAs regulating mitofilin in the future would provide an ideal therapeutics for improving mitochondrial structure, function and ultimately cardiac function in T1DM.

Antagomirs currently utilized for miRNA blockade are systemically delivered and are not targeted towards a specific organelle. Presence of a mitochondrial targeting sequence in an alpha myosin heavy chain driven construct utilized to deliver antagomir would allow its import specifically inside the cardiac mitochondrion. Proper import of the antagomir specifically into the cardiac mitochondrion would further increase its mitomiR blockade and provide efficient therapeutics targeted towards cardiac mitochondrial proteins. Mitochondrial targeting sequence are particular amino acid sequences that are essential for proper import of pre-proteins in the mitochondria (18, 20). In summary, future research endeavors geared towards delivery of antagomirs targeted to the cardiac mitochondria in order to block miRNAs regulating mitofilin would provide an ideal therapeutics for T1DM. Restoration of mitochondrial structure through mitofilin via antagomir approach in type 1 diabetic heart would improve mitochondrial function and ultimately cardiac function, one of the key findings of this dissertation.

Another potential way of site specific antagomir delivery would be the utilization of catheters. A catheter system, comprised of distensible penetrating element with a distally located chamber for holding therapeutic agent, is used to inject large molecules into the body. This delivery system can be tissue specific including the heart, pancreas, stomach, esophagus, large intestine and other tissues thus allowing delivery of drugs to the sites where there are more

needed (2). Thus, injection of antagomirs to block miRNAs regulating mitofilin utilizing the catheter system would also provide an ideal therapeutics for T1DM.

Decrements in content of mitofilin have been reported in many human diseases including Down's syndrome (3, 16), Parkinson's disease (21, 22), epilepsy (6, 17) and neurodegeneration (25, 26). With mitofilin providing cardioprotection in type 1 diabetic heart through maintenance of mitochondrial morphology and function, it warrants future studies designed to investigate the role of mitofilin in the above mentioned pathologies. Further, it invites other investigators to examine the role of mitofilin in pathologies like aging (12) where the IFM subpopulation is predominantly affected.

Mitofilin is a critical component of the MICOS complex comprised of proteins like Mio10, Aim5, CHCHD3, and MOMA-1 where it functions as a central organizer of mitochondrial architecture and cristae morphology (1, 8, 9, 19, 23, 27). Studies focused on assessing these other proteins of the MICOS complex in diabetic cardiomyopathy as well as other pathologies with mitochondrial morphological abnormalities will allow a better understanding of the role of mitofilin and the MICOS complex as a whole thus further strengthening MICOS and its components as potential therapeutic targets in many mitochondrial pathologies.

Presence of microRNAs in the mitochondrion provides a novel platform to investigate their role in diabetic cardiomyopathy. As reported in chapter 4 of this dissertation miRNAs alterations could potentially regulate mitochondrial genome encoded proteins in type 1 diabetic heart. Similar studies centered towards assessment of mitochondrial miRNAs in type 2 diabetic hearts should be undertaken. Findings from these studies will provide a comprehensive as well as

common list of miRNAs that contribute towards the pathogenesis of DM. Not only will this allow pinpointing common miRNAs contributing towards both type 1 and type 2 DM, it will provide other investigators a novel research area and focus. Findings of chapter 4 with respect to the role miR-378 in regulating ATP6 should initiate other key studies focusing on other mitochondrial as well as nuclear proteins.

In summary, findings derived from the studies in this dissertation has the potential to initiate novel research and projects geared towards examining the role of mitofilin and miRNAs in proper functioning of mitochondria in various physiological and pathological conditions. Also, the reported findings can be highly impactful within the scientific community as it presents novel avenues for therapeutic targets in T1DM.

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outer membrane interactions differentially depend on mitofilin domains. *J Mol Biol* 422: 183-191.

CURRICULUM VITAE

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EDUCATION

West Virginia University (Morgantown, WV)	2009 – Present
School of Medicine; Division of Exercise Physiology	
Center for Cardiovascular and Respiratory Sciences	
Biomedical Health Sciences; Ph.D. Candidate	2013-Present
Morgan State University (MSU) (Baltimore, MD)	2007 – 2009
Bachelor of Science in Biology	

Baltimore City Community College (Baltimore, MD)

2005-2007

Associates Degree in Biotechnology

DISSERTATION

Type 1 Diabetic Heart: Examination of mitochondrial structure and MicroRNAs

Mentor: Dr. John Hollander

Committee Members: Stephen Alway, Albert Berrebi, Mark Olfert, and Emidio Pistilli

HONORS and AWARDS

Panelist for Annual Graduate Science Career Workshop

2012

MBRS-RISE program, MSU

Student judge for Appalachian Regional Cell Conference (ARCC)

2012

Caroline tum Suden/Frances Hellebrandt Professional

2012

Opportunity Award, Experimental Biology Conference

WVU School of Medicine Biomedical Sciences Travel award

2012

Tamika Price memorial Award for academic achievement(MSU)

2009

Academic Excellence Award, MSU

2009

1 st place in Chemical Sciences at 2008 annual HBCU-UP Undergraduate research conference, Atlanta	2008
Dean's list recognition and recipient of Certificate of Excellence MSU (Graduated with highest GPA in School of Computer, Mathematical and Natural Sciences)	2007 – 2009
Community College Honors Scholarship at MSU	2007 – 2009
Distinguished scholar at Baltimore City Community College (Dean's List)	2005 – 2007

PROFESSIONAL SOCIETIES

Member, American Heart Association; WVU	2012 – 2013
Member, American Physiology Society; WVU	2010 – 2013
Center for Cardiovascular and Respiratory Sciences; WVU	2010 –Present
Student member of NOBCHHE (The national organization for professional Advancement of Black Chemists and Chemical Engineers)	2007-2009
The National Scholars Honor Society	2012

RESEARCH SUPPORT

WVU School of Medicine Predoctoral Fellowship

2009 – 2014

AHA Predoctoral Fellowship (Submitted winter 2013; not funded)

2013

Average Priority Score:

1.92

Percentile Rank:

32.41%

PRESENTATIONS

Thapa D, Nichols CE, Shepherd DL, and Hollander JM. Overexpression of mitofilin restores cardiac contractile function during a type 1 diabetic insult. *Van Liere Convocation, West Virginia University. 2014*

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Thapa D, Baseler WA, Croston TL, Lewis SE, Hollander JM. Examination of microRNA (miRNA) dysregulation in the type 1 diabetic heart and its functional implications. *FASEB J. 2011 25:lb464*

Thapa D, Baseler WA, Croston TL, Lewis SE, Hollander JM. MicroRNA Dysregulation and Functional Implications on Mitochondrial Proteomes in Type 1 Diabetic Heart. *Van Liere Convocation, West Virginia University. 2011*

PUBLICATIONS

Articles

Thapa D*, Jagannathan R, Nichols CE, Shepherd DL, Croston TL, Lewis SE, Hollander JM. Novel association of mitofilin with ATP synthase subunits in mitochondrial subpopulations. In Preparation (2015)

Thapa D*, Nichols CE, Lewis SE, Shepherd DL, Jagannathan R, Croston TL, Tveter KJ, Holden AA, Baseler WA, Hollander JM. Transgenic overexpression of mitofilin attenuates diabetes mellitus-associated cardiac and mitochondria dysfunction. [J Mol Cell Cardiol.](#) 2014 Nov 22. pii: S0022-2828(14)00360-5. doi: 10.1016/j.yjmcc.2014.11.008. [Epub ahead of print]

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Shepherd DL, Croston TL, Nichols CE, **Thapa D**, Lewis SE, Dick GM, McLaughlin S, Hollander JM. Longitudinal assessment of type I diabetes mellitus using conventional echocardiography and speckle-tracking based strain imaging. In Preparation (2015)

Nichols CE, Baseler WA, **Thapa D**, LaFata GL, Croston TL, Shepherd DL, Lewis SE, Knuckles TL, McCawley M, Nurkiewicz TR, Hollander JM. Effect of mountain-top mining particulate exposure on mitochondria function and apoptotic propensity. In preparation (2015)

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Hollander JM, **Thapa D**, Shepherd DL. Physiological and Structural Differences in Spatially-Distinct Subpopulations of Cardiac Mitochondria: Influence of Pathologies. *Am J Physiol Heart Circ Physiol*. 2014 Jul 1;307(1):H1-14. Review. PMID: 24778166

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Glutathione Peroxidase Am J Physiol Regul Integr Comp Physiol. 2013 Apr 1;304(7):R553-65
doi:10.1152/ajpregu.00249.2012. Epub 2013 Feb 13. PMID: 23408027

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Baseler WA, Dabkowski ER, Jagannathan R, **Thapa D**, Nichols CE, Shepherd DL, Croston TL, Schnell DM, Hollander JM. Overexpression of phospholipid hydroperoxide glutathione peroxidase (MPHGPx) attenuates cardiac mitochondrial proteomic loss and reverses protein import detriments observed with type 1 diabetes mellitus. *FASEB J. 2012 26:1127.4*

Fancher IS, Baseler WA, Croston TL, **Thapa D**, Shepherd DL, Nichols CE, Lewis SE, Jagannathan R, Asano S, Dick GM, Hollander JM. Differential expression of mitoKATP subunits in cardiac mitochondrial subpopulations and the influence of Type I diabetes. *FASEB J.* 2012 26:1057.6

Lewis SE, Baseler WA, Croston TL, **Thapa D**, Jagannathan R, Hollander JM. Type 1 diabetes mellitus differentially regulates mitochondrially-encoded proteins in cardiac mitochondrial subpopulations. *FASEB J.* 2012 26:1b748

Jagannathan R, Baseler WA, **Thapa D**, Croston TL, Shepherd DL, Nichols CE, Hollander JM. HDAC6 regulates mitochondrial oxidative phosphorylation by ATP synthase β subunit acetylation in diabetic cardiomyopathy. *FASEB J.* 2012 26:869.13

Nichols CE, Baseler WA, **Thapa D**, LaFata GL, Croston TL, Shepherd DL, Lewis SE, Knuckles TL, McCawley M, Hendryx M, Nurkiewicz TR, Hollander JM. Mountain-top mining particulate matter exposure increases markers of mitochondrially-driven apoptosis in rat cardiac tissue. *FASEB J.* 2012 26:1036.15

Shepherd DL, Croston TL, McLaughlin SL, Baseler WA, Nichols CE, **Thapa D**, Lewis SE, Hollander JM. Longitudinal assessment of type I diabetes mellitus using conventional echocardiography and speckle-tracking based strain imaging. *FASEB.* 2012 26:1054.11

Thapa D, Baseler WA, Jagannathan R, Dabkowski ER, Croston TL, Nichols CE, Shepherd DL, Lewis SE, Hollander JM. miRNA-141 is a potential regulator of the mitochondrial phosphate carrier (slc25a3) in the type 1 diabetic heart. *FASEB J.* 2012 26:869.11

Audet G, Fulks D, **Thapa D**, Olfert I. M. Manipulation of TSP-1 and the CD36 pathway results in changes in skeletal muscle mitochondrial enzyme activity. APS: Integrative Biology of Exercise. October, 2012

Nichols C.E., Baseler W.A., **Thapa D.**, LaFata G.L., Croston T.L., Shepherd D.L., Lewis S.E., Knuckles T.L. McCawley M., Hendryx M., Nurkiewicz T.R., Hollander J.M. Effect of mountain-top mining particulate exposure on function and apoptotic propensity of distinct subcellular species of mitochondria. AESOT. April 2012.

Nichols C.E., Shepherd D.L., Croston T.L., **Thapa D.**, Lewis S.E., Jinghai Y., McBride C., Nurkiewicz T.R., Hollander J.M. Acute inhalation exposure of titanium dioxide nanoparticles induces diastolic dysfunction and mitochondrial dysfunction in mice. AESOT. April 2012.

Thapa D, Baseler WA, Jagannathan R, Hollander JM. Mitochondrial phosphate carrier (slc25a3) is regulated by miRNA-141 in the type 1 diabetic heart. Van Lier Convocation, West Virginia University. February 2012

Croston TL, Shepherd DL, Baseler WA, Dabkowski ER, **Thapa D**, Nichols CE, Jagannathan R, Lewis SE, Hollander JM. Influence of diabetic cardiomyopathy on cardiolipin biosynthesis. Van Liere Convocation, West Virginia University. February 2012.

Shepherd DL, Croston TL, Nichols CE, Baseler WA, **Thapa D**, Hollander JM. Conventional echocardiography and speckle-tracking based strain imaging of the type 1 diabetic heart. Van Liere Convocation, West Virginia University. February 2012.

Nichols C. E., Baseler W.A., **Thapa D.**, LaFata G.L., Croston T.L., Shepherd D.L., Lewis S.L., Knuckles T.L., McCawley M., Hendryx M., Nurkiewicz T.R., Hollander J.M. Effect of mountain-top mining particulate exposure on mitochondria function and apoptotic propensity. Van Liere Convocation, West Virginia University. February 2012.

Croston TL, Baseler WA, **Thapa D**, Lewis SE, Nurkiewicz TR, Yi J, Hollander JM. Influence of Nanoparticle Inhalation on Cardiac Mitochondrial Function. *FASEB J. 2011 25:1b561*

Baseler WA, Dabkowski ER, **Thapa D**, Croston TL, Williamson CL, Hollander JM. Proteomic alterations of distinct mitochondrial subpopulations in the type 1 diabetic heart: Contribution of protein import dysfunction. *FASEB J. 2011 25: 825.6*

Dabkowski ER, Baseler WA, Croston TL, **Thapa D**, Hollander JM. Mitochondrial phospholipid hydroperoxide glutathione peroxidase (mPHGPx) overexpression preserves the inner mitochondrial membrane in the diabetic heart. *FASEB J.* 2011 25:1095.5

Thapa D, Baseler WA, Croston TL, Lewis SE, Hollander JM. Examination of microRNA (miRNA) dysregulation in the type 1 diabetic heart and its functional implications. *FASEB J.* 2011 25:1b464

Croston TL, Baseler WA, **Thapa D**, Nurkiewicz TR, Hollander JM. Examination of Mitochondrial Function Influenced by Environmental Nanoparticle Exposure. Van Liere Convocation, West Virginia University. 2011

Thapa D, Baseler WA, Croston TL, Lewis SE, Hollander JM. MicroRNA Dysregulation and Functional Implications on Mitochondrial Proteomes in Type 1 Diabetic Heart. Van Liere Convocation, West Virginia University. 2011

REFERENCES

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